# A screen for interactors of Lkb1 and a role for Lkb1 in the regulation of polarity in the *Drosophila* eye

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**I, *Afifa Sulaiman Khan*, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.**

# Abstract

In humans, loss of function mutations in the Lkb1 serine-threonine kinase are associated with Peutz-Jegher syndrome, an inherited cancer predisposition syndrome. Roles for Lkb1 have been described in various processes, including cell cycle regulation, apoptosis and cell polarity. Comparatively little is understood of the role of Lkb1 in regulating epithelial cell polarity.

The establishment and maintenance of apicobasal polarity is a fundamental process that occurs at different stages throughout development. The Lkb1 kinase has been shown to play a conserved role in regulating epithelial cell polarity in *C. Elegans*, *Xenopus*, *Drosophila* and mammals. The primary focus of this thesis has been to further characterise the polarity phenotype and mechanisms by which Lkb1 may regulate epithelial polarity in *Drosophila*.

To study the role of Lkb1 in the control of epithelial polarity, I generated loss of function clones in the *Drosophila* neuroepithelium, the eye. Immunohistochemical and biochemical analysis of *lkb1* clones in the eye reveal that Lkb1 is required for the restriction of apical, junctional and basal determinants to their appropriate domains, for the correct formation of adherens junctions, and for the maintenance of photoreceptor cell morphology.

I further demonstrate that in the absence of Lkb1, the β-catenin homologue Arm and the polarity determinant Par-1 accumulate, and additionally, that Par-1 shows reduced phosphorylation at a site that regulates its localisation and activity. Genetic interactions assays provide further evidence that the pleiotropic effects of *lkb1* loss of function may be mediated through the misregulation of Par-1 and Armadillo.

I have also conducted a small-scale modifier EMS screen in *Drosophila* to isolate components of the Lkb1 pathway. We have screened approximately 9,500 flies, and recovered a number of mutants, which can now be mapped and identified using a multi-tiered approach.

The role of Lkb1 in regulating cell polarity is conserved from *Drosophila* to humans. Thus, a deeper understanding of the mechanisms underlying the Lkb1 mediated regulation of epithelial polarity may make a valuable contribution to, and provide a strong basis for progress in the treatment of Peutz-Jegher syndrome.

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This thesis is dedicated to the memory of Ruksana Yasmin, my best friend.

## Abbreviations

| | |
|---|---|
| AJ | Adherens Junctions |
| BrdU | 5-bromo 2' deoxyuridine |
| β-Me | Beta-mercaptoethanol |
| c.c | Cone cells |
| c.c.p | Cone cell plate |
| GFP | Green Fluorescent Protein |
| IPTG | Isopropyl-β-D-Thiogalacto-Pyranoside |
| IRS | Inter-rhabdomeral space |
| MOPS | 3[N-morpholino]propane-sulphonic acid |
| NGS | Normal Goat Serum |
| PAGE | Polyacrylamide gel electrophoresis |
| PFA | paraformaldehyde |
| PBS | phosphate buffered saline |
| PB | phosphate buffer |
| pd | pupal development |
| PRC | Photoreceptor cell |
| SAR | sub-apical region |
| SEM | Scanning electron microscopy |
| SPB | sodium phosphate buffer |
| TEM | Transmission electron microscopy |
| ZA | Zonula adherens |

# 1 Introduction

## 1.1 Peutz-Jegher syndrome

The hallmark of Peutz-Jegher Syndrome (PJS), an autosomal dominant cancerous disorder, is the combination of gastrointestinal hamatomatous polyps with the abnormal pigmentation of the buccal mucosa. PJS patients also suffer from an increased incidence of cancer, including lung, breast, and testicle cancer, and the overall cancer risk was estimated to be 18-fold increase to that found in the general population (Giardiello et al., 1987; Hearle et al., 2006). Identified in 1921, PJS has since been the subject of both clinical studies detailing the histology and incidence of the disease, as well as functional studies in a number of model organisms, examining the underlying molecular basis for PJS.

Hamartomas are typically defined as benign growths that are composed of multiple well differentiated tissues that are endogenous to the site of origin. Histological studies of PJS polyps demonstrated that they are large, pedunculated structures, that are composed of smooth muscle that originates from the muscle stalk and well differentiated epithelial tissues. Additionally, hyperplastic and adenomatous polyps are also sometimes observed in PJS patients [Fig. 1.1].

In 1997, linkage studies demonstrated that mutations in the LKB1 gene were responsible for PJS. LKB1 is a 433-residue serine-threonine kinase, and the majority of germline mutations in PJS families were found to be truncations or point mutations that compromised LKB1 kinase function [Fig. 1.2]. The *LKB1* gene consists of 10 exons, the first of which is partially coding, and the last non-coding and *LKB1* mRNA is expressed ubiquitously.

The question of whether LKB1 is a recessive tumour suppressor and requires biallelic inactivation in order to initiate polyp formation has thus far not been resolved. Independent studies, employing different techniques such as *in situ* hybridisation, and micro-satellite mapping have yielded disparate results, and alternative theories have suggested that PJS polyps may be haploinsufficient for LKB1.

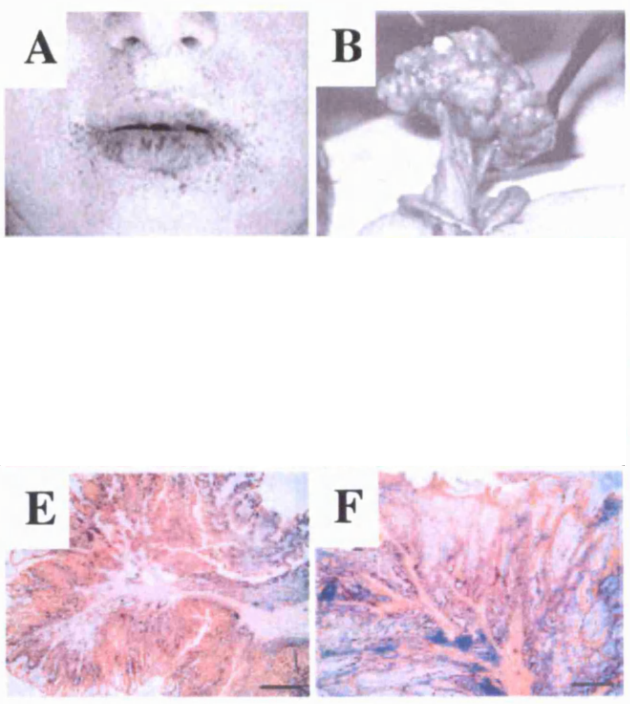



**Fig. 1-1 PJS polyps in humans and mice.** (A) External PJS symptoms include pigmentation of the buccal mucosa (B) PJS patients exhibit multiple hamartomas throughout the GI tract (C-F) Ultrastructure of mouse and human polyps (C) Hematoxylin eosin staining of cross sections taken from resin embedded glandular stomach of *lkb1* mice showing 3 small hamartomatous polyps. Arrows show the smooth muscle core that is contiguous with the muscularis mucosa. (D) Higher magnification of the boxed region in C, showing a well developed smooth muscle component, which creates a lattice framework for glandular epithelia (E and F) Hematoxylin eosin staining of a murine polyp (E) and human PJS polyp (F) showing a striking similarity, particularly in the smooth muscle component (C and D taken from Rossi *et al*, 2002).

## 1.2 Lkb1

Functional studies of LKB1 have since identified roles for LKB1 in diverse biological processes. In addition to classical tumour suppressor roles such as the control of cell cycle and apoptosis, other functions have also been described, such as the control of metabolic kinases, and the regulation of cell polarity.

A high degree of sequence homology between LKB1 and its putative orthologues in other species suggests that functionality may also be conserved. To date, orthologues have been identified and studied in *Caenorhabditis elegans (par-4), Xenopus laevis (XEEK1), Drosophila Melanogaster* (*Lkb1*), and mouse *Mus musculus* (*LKB1*). Mouse and human LKB1, and XEEK1 share a conserved nuclear localisation signal (NLS), and LKB1 can be observed both in the cytoplasm and the nucleus (Tiainen et al., 2002).

The kinase domain is highly conserved in LKB1 orthologues. Although the amino- and carboxy-terminal regions are not as well conserved, other conserved motifs include the C-term prenylation motif, shown to be required both in mammalian systems and in *Drosophila* for localisation to the membrane (Collins et al., 2000; Martin and St Johnston, 2003; Sapkota et al., 2001), and a conserved RKLS consensus phosphorylation site near the C terminus. In both mammalian and *Drosophila* Lkb1, this site can be phosphorylated in a PKA dependent manner, and is required to regulate cell growth in cell culture systems, and to modulate Lkb1 activity in *Drosophila*

(Collins et al., 2000; Martin and St Johnston, 2003; Sapkota et al., 2001) [Fig 1.2].

The deregulation of Wnt signalling is known to play a key role in human colorectal tumorigenesis. Mutations in the *Apc* gene underlie approximately 80% of sporadic colorectal cancers, and the loss of *Apc* was found to activate Wnt signalling through the nuclear accumulation of β-catenin (Sansom et al., 2004). As a result, there has been some interest in the Wnt signalling pathway in PJS. A study of the LKB1 orthologue, XEEK1 in *Xenopus* demonstrated that loss of XEEK1 could lead to axis formation defects that phenocopied an aberrant Wnt signalling phenotype (Ossipova et al., 2003). Further analysis showed that XEEK1 could associate with protein kinase C-ζ (PKC-ζ) and GSK3β, and regulate the PKC-ζ mediated phosphorylation of GSK3β - in this way potentiating Wnt signalling. By contrast, a separate study looking at mammalian LKB1 in a cell culture system demonstrated the opposite result, that LKB1 could associate with Par-1A, a Dishevelled (Dvl) associated kinase, and prevent Par-1A mediated potentiation of Wnt signalling by redirecting its activity to other substrates (Spicer et al., 2003). Interestingly, a study by Martin *et al* suggested that in *Drosophila,* Lkb1 is downstream of Par-1 (Martin and St Johnston, 2003).

Most recently, work by Wang *et al* showed that Lkb1 was required in *Drosophila* for the activation of Par-1 and indirectly the phosphorylation of tau, a microtubule binding protein that is associated with neurodegenerative disorders. They showed that Lkb1 could phosphorylate Par-1 on its activation loop, on a residue that is critical for its activity, and was thus required for Par-1 activation (Wang et al., 2007a). This modification of Par-1 is consistent with

studies conducted in mammalian systems that showed that LKB1 could phosphorylate mammalian PAR-1 on its activation loop (Lizcano et al., 2004).

Tiainen *et al* showed that reintroducing LKB1 into tumour cell lines (human G361 melanoma cells) that lacked LKB1 could result in a G1 cell cycle arrest (Tiainen et al., 1999). Further evidence for a role for LKB1 in cell cycle control was demonstrated by Marignani *et al*, who showed that LKB1 could interact with Brg1, a chromatin remodelling complex, and was necessary for Brg1 dependent growth arrest in SW13 cells (Marignani et al., 2001).

A role for LKB1 in apoptosis was also demonstrated by two groups, who showed that LKB1 could associate with and phosphorylate p53 *in vitro*, and potentiate p53 dependent pathways (Karuman et al., 2001; Zeng and Berger, 2006). A report detailing the phenotype of *lkb1-/- p53 -/+* knock out mouse showed that lack of p53 causes the earlier onset of gastric hamatomas and hepatic tumorigenesis (Takeda et al., 2006).

Other mouse models of LKB1 have shown that *lkb1* mice develop gastrointestinal polyps that are histologically indistinguishable from human PJS polyps (Bardeesy et al., 2002; Rossi et al., 2002) [Fig. 1.1]. In addition, *lkb1* mice displayed severe developmental retardation, and were embryonic lethal, indicating that LKB1 has an essential role in embryogenesis (Bardeesy et al., 2002; Jishage et al., 2002).

Many studies have also attempted to describe the genetic response to loss of LKB1. Microarray studies have been carried out e.g. in lung cancers that lack LKB1; among the findings were the deregulation of the Wnt pathway (Lin-Marq et al., 2005), and up regulation of PTEN, suggesting that LKB1 may be involved in regulating the PTEN/PI3K pathway (Jimenez et al., 2003).

Other affected genes related to regulation of the cytoskeleton (e.g. MMP17), adhesion (e.g. cadherins and other adhesion molecules), signal transduction and growth factors, apoptosis, and cell cycle control (Fernandez et al., 2004) (Fernandez et al., 2004; Jimenez et al., 2003).

Recent activity in the field of LKB1 studies has centred on its role in regulating the metabolic stress kinase family, 5'-AMP activated protein kinase (AMPK). The yeast orthologue of AMPK is Snf1, and upstream kinases Elm1, Tos3 and Pak1 show sequence similarity to LKB1 (Hong et al., 2003a; Woods et al., 2003). LKB1 was shown to phosphorylate and activate AMPK (Woods et al., 2003), and thus regulate energy homeostasis processes. LKB1 has been shown to also regulate 11 of 12 AMPK kinases *in vitro* in mammalian studies, further illustrating that LKB1 has a role in regulating AMPK signalling (Lizcano et al., 2004).

Since its discovery, many studies have attempted to describe the downstream effectors of LKB1, however, less work has been done on upstream regulators. Work by Boudeau *et al* showed that LKB1 may require accessory proteins to function at full capacity. Two important co-adaptors were identified for LKB1 that were shown to be critical for LKB1 function and localisation. STE20 related adaptor (STRAD) and scaffolding protein MO25 were identified as co-adaptors that can bind, activate and translocate LKB1 (Baas et al., 2003; Boudeau et al., 2003; Hawley et al., 2003).

Finally, two studies, one in *Drosophila* and one in mammalian cells demonstrated that LKB1 has a conserved role in regulating cell polarity. The control of cellular polarity involves signalling pathways that are conserved from yeast to humans. A screen to discover regulators of anterior-posterior (A-

P) polarity in the *Drosophila* oocyte identified Lkb1, and showed that Lkb1 has a role in the generation of A-P polarity in the oocyte, as well as in the repolarisation of the microtubule cytoskeleton that occurs prior to embryogenesis (Martin and St Johnston, 2003). Additionally, Lkb1 was shown to function in concert with Par-1 to regulate A-P polarity, since loss of *par-1* or *lkb1* result in similar phenotypes in the oocyte, and the overexpression of Lkb1 was able to partially rescue the A-P polarity phenotype in *par-1* mutants. Martin *et al* also demonstrated a more general role in the regulation of epithelial polarity in *Drosophila* follicle cells.

Baas *et al* showed that LKB1, when activated with its co-adaptor STRAD into intestinal cells that lacked LKB1, could induce a complete polarisation of the cell (Baas et al., 2004). They found that upon induction of Lkb1 in epithelial cells lacking Lkb1, cells underwent rapid remodelling of the cytoskeleton, as well as generalised resorting of polar markers. Remarkably, this complete polarisation of the cell could be observed in single cells suspended in cell culture. The results of this study were unexpected, since contrary to prevailing theory, which suggests that junctions act as a primary cue and landmark for the polarisation of the cell, these cells were able to segregate both apical and basolateral markers in the absence of junctional cell-cell contacts, and in a cell-autonomous fashion.

**Fig. 1.2 Schematic of human LKB1 protein** (A) Mutations in the LKB1 gene in PJS patients occur predominantly in the kinase domain, and other mutations are predicted to be loss of function mutations. Small deletions or point mutations are indicated in black, mutants that lack autophosphorylation activity are in red, and a mutant that has been shown to lead to reduced autophosphorylation activity is in blue. 2 putative NLS sites are indicated in green, phosphorylation sites in red, including the conserved RKLS site at Ser431, and a conserved prenylation site in blue (adapted from Yoo *et al,* 2002).

### *1.2.1* Lkb1 in *Drosophila Melanogaster*

The previous studies demonstrated that *Drosophila* and human LKB1 share conserved roles in regulating cell polarity. The *Drosophila* orthologue is strongly conserved in the kinase domain, showing 66% identity, and 81% similarity in this domain with human LKB1. Although the amino and carboxy terminals are not well conserved, a C term prenylation motif, necessary for the cortical localization of LKB1, and an RKLS consensus phosphorylation site, shown to be phosphorylated *in vivo* and *in vitro* by PKA, are conserved in mammalian cells and in *Drosophila* (Collins et al., 2000; Martin and St Johnston, 2003; Sapkota et al., 2001).

The complexes that regulate epithelial polarity in *Drosophila* are conserved in diverse species, from yeast to humans (discussed further in section 1.5). The interactions and underlying mechanisms of these complexes, as well as the structure of these proteins are extremely well conserved, and as such, *Drosophila* is an excellent model organism to study these processes.

Biological processes such as proliferation, differentiation, apoptosis and polarity are also well studied in *Drosophila* tissues such as the retina, and the retina in particular is an excellent system in which to study the processes of cellular polarity, since it exhibits many different levels of organisation, from apicobasal polarity to planar cell polarity.

## 1.3 Epithelial polarity

The polar distribution of proteins and lipids in epithelial cells are essential for their specialised functions, and the signalling mechanisms that lead to the development of polar domains, with distinct protein and lipid identity are processes that are conserved from invertebrates to vertebrates (reviewed in (Nelson, 2003)).

The establishment of polarity is a complex process, and one that is thought to involve multiple cues from the extracellular matrix (reviewed in (Small and Kaverina, 2003)), as well as cues from neighbouring cells in the form of junctional contacts (reviewed in (Perez-Moreno et al., 2003)). In addition to external cues, internal mechanisms for the establishment of polarity involve signalling cascades and complex protein-protein interactions (reviewed in (Nelson, 2003)). These distinct mechanisms bring together an intricate program of polarity that allows cells to both polarise individually and also to coordinate themselves within the wider context of the tissue.

The basic features of epithelial cell polarity are the formation of apical, basal and lateral surfaces. Each domain is specialised for different functions, for instance, the apical domain, since it faces the topological outside of the organism, is commonly involved in protection and secretion processes. The basal domain is involved in communication and the sharing of components with other cells. The lateral domain is involved in cell-cell contact, and it is from here that junctions mediate molecule movement and restrict the lateral movement of proteins ( reviewed in(Drubin, 2000)) [Fig. 1.3].

Studies of epithelial cell lines have demonstrated that cell-cell contact can induce cell polarity in previously unpolarised cells (Adams et al., 1996; Vasioukhin et al., 2000). Adherens junctions are thought to act as a principal landmark for cell polarity, acting as a physical point at the membrane that demarcates the apical domain from the basal domain, and also having a more active role in preventing the diffusion of apical and basal proteins into other domains.

**Fig. 1.3 Mammalian and invertebrate apicobasal polarity** (A) The polarised architecture of the cell is well conserved in mammalian and invertebrate cells. (B) Polarised complexes, and their interactions in mammalian and invertebrate cells are also closely conserved (A and B adapted from Ohno *et al,* 2006*)*

## 1.4 Stages of *Drosophila* eye development

Since the work described in this thesis was conducted mainly in the larval, pupal and adult eye, I will focus first on the development of the *Drosophila* eye, and later, on the polarity determinants that regulate polarity in the eye.

The *Drosophila* larval eye disc is a columnar epithelium, with simple apicobasal polarity. During pupal development, this simple epithelium undergoes dramatic remodelling of tissue structure and redefines its axis of polarity, leading to a complex crystalline neuro-epithelium that is the adult retina.

The adult retina is comprised of 800 repeating units, called ommatidia. Each ommatidium is composed of 8 photoreceptor cells (PRCs) and 12 accessory cells, which are packed in a stereotypical manner to form a regular hexagonal array.

The development of the *Drosophila* eye imaginal disc can be roughly divided into 2 stages: Firstly, a period of exponential growth during which the animal accrues a mass of cells that will eventually form the imaginal disc. This regulated growth occurs during the first and second instars. The eye disc undergoes extensive proliferation during this stage, from a precursor of 10-30 cells to reach a final size of nearly 50,000 cells. Since organ size is primarily determined by cell proliferation, this process is tightly regulated and signalling pathways that control proliferation include the morphogens decapentaplegic (Dpp), wingless (Wg) and Hedgehog (Hh). The second stage of development is

commenced by the progression of a morphogenetic furrow (MF) across the eye imaginal disc that initiates differentiation and patterning in the disc, organising the disc into what can be recognised as the presumptive eye.

During pupal development (pd), the imaginal eye disc undergoes a process of morphogenesis and structural elaboration that may include a repolarisation of the retinal photoreceptor cells (PRCs). Ommatidial preclusters undergo a 90° rotation that turns the apices of the PRCs towards each other. Once PRCs are fixed in position - by the formation of new contacts with the retinal floor at the feet of the PRCs, and with the lens secreting cone cells at the top of the PRCS - the retina finally undergoes a dramatic increase in depth along the proximo-distal axis. This leads to a deepening of the retinal epithelium and an expansion of the membrane domains along this axis.

### 1.4.1 The specification of retinal cell types begins in the third instar eye disc

During the growth phase of the larval disc, cell cycles are asynchronous and dispersed throughout the disc. Differentiation begins in the posterior, with the initiation of the MF by Hh signalling. The MF is a wave of differentiation that sweeps through the disc, from the posterior to the anterior of the disc, until all cells are differentiated, and organised into ommatidial pre-clusters [Fig. 1.4]. It is during the third instar that cells in the undifferentiated disc become recruited to the presumptive ommatidia as specific PRCs or accessory cells.

Cells anterior to the furrow continue to proliferate asynchronously – as the MF progresses through the disc, cells arrest in the G1 cycle, and begin to

commit to cell fates. The neural cells, PRCs R1-R8 are specified first. Of these, the R8 is the first cell to be committed, and subsequent signalling from the R8 recruits a further 4 PRCs, R2-R5. The remaining cells then exit the G1 arrest and undergo one final S phase, in order to generate the remaining PRCs as well as the accessory and cone cells. The R7 is the last of the PRCs to be specified. The patterning of PRCs follow a fixed chain of events, and the timing of differentiation can be accurately followed by counting the number of rows posterior to the MF. Approximately 9 rows posterior to the MF, ommatidial precursors have recruited their full complement of 8 PRCs and 4 cone cells.

The cone cells are specified after the PRCs, followed by the pigment cells. Programmed cell death subsequently eliminates supernumerary cells in the early pupal retina, shortly after complete ommatidial recruitment at 20% pd (Cagan and Ready, 1989; Wolff and Ready, 1991), and this apoptosis is restricted to cells that are not in direct contact with the cone cells and the primary pigment cells (Miller and Cagan, 1998).

The specification of different PRC subtypes is achieved by the differential expression of transcription factors e.g. BarH1 in R1 and R6 cells (Higashijima et al., 1992), and Prospero in R7 cells (Kauffmann et al., 1996).

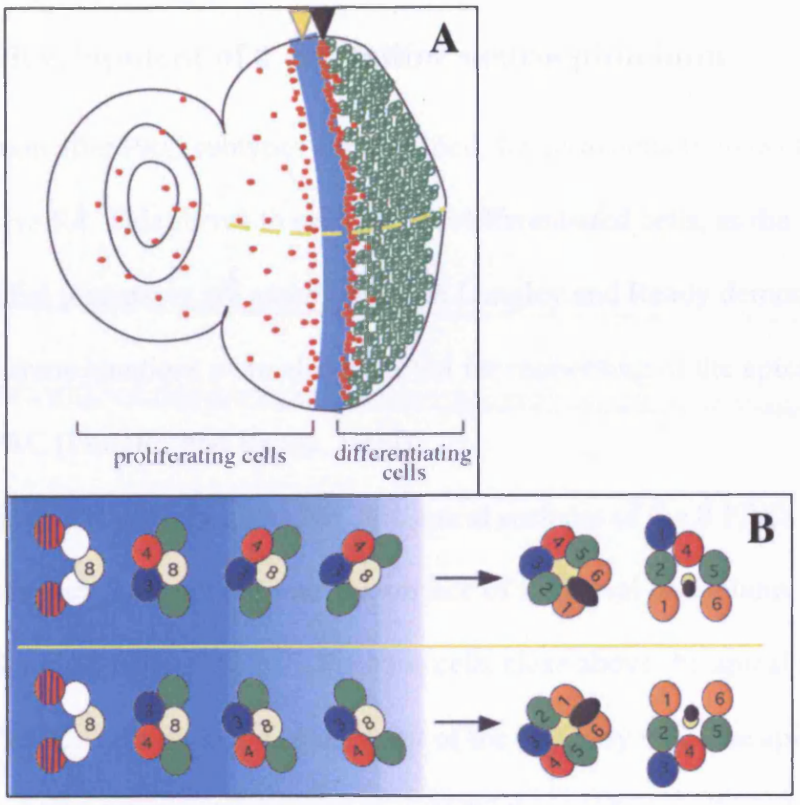



**Fig. 1.4 Eye disc development in the larva** (A) Schematic of the larval eye disc. The position of the morphogenetic furrow (MF) is indicated by a black arrowhead, and the second mitotic wave is indicated by a yellow arrowhead. In this figure, the MF has traversed half of the eye field. Anterior is to the left, posterior to the right. Posterior to the MF are differentiating cells, and developing proneural clusters. Successive cycles of cell fate induction have led to the specification of the photoreceptors, illustrated in (B). Anterior to the MW are asynchronous proliferating cells. (Figure used with permission from Elizabeth Silva) (B) Assembly of the ommatidia precursor. The R8 is the first PRC to be recruited. Following the stepwise recruitment of the remaining PRCs, the precluster undergoes a 90° rotation, and is reassembled into the stereotypical trapezoid configuration of PRCs that is seen in the adult retina.

### 1.4.2 Elaboration of the adult *Drosophila* retina leads to the development of a crystalline neuroepithelium

Soon after PRC subtypes are specified, R8 forms junctions with all other PRCs save R4. This serves to stabilise the differentiated cells, as the ommatidial precursors are assembled, and Longley and Ready demonstrated that adherens junctions were also essential for reorienting of the apical surfaces of the PRC (Longley and Ready, 1995).

Up to 9 rows behind the MF, the apical surfaces of the 8 PRCs and 4 cone cells thus far specified face the surface of the larval epithelium. Between rows 18 and 22 behind the MF, the cone cells close above the apical surfaces of the PRCs, resulting in a displacement of the PRCs by 90°. The apical surfaces of the PRCs subsequently face each other, and create a pocket of space that later develops into the inter-rhabdomeral space (IRS). The 4 cone cells establish new contacts with each other during this process to adopt a cross like arrangement; this forms the cone cell roof.

Pigments cells also undergo cell shape changes that result in two concentric arrangements of cells around the inner cone cell cluster: two large cells form the primary pigment cells that encase the cone cell clusters. Adjacent to this, the secondary and tertiary pigment cells, and the inter-ommatidial bristle cells make up the second concentric circle of cells, which also form the hexagonal pattern of the retina.

Throughout the involution of the disc, adherens junctions between PRCs are maintained, allowing changes in cell morphology and at the same time

maintaining the integrity of the precluster as a unit. Between 37 and 55% pd, the PRC apical surfaces undergo an expansion towards the retinal floor. AJs between PRCs are maintained until approx 55% pd, when new AJs are formed at the cone cell plate, a structure formed by the feet of the cone cells, at the base of the ommatidia.

Junctional rearrangements between PRCs occur soon after this anchoring process, which serve to stabilise the new positions of the PRCs. In the ommatidial precluster in the imaginal disc, R8 occupies a central position in the cluster, and shares AJ contacts with all of the PRCs except R4. As the apical surfaces are anchored and PRCs are stabilised to the retinal floor, contacts between R8/R3, R8/R5 and R8/R6 are lost. In this way, the cone cell plate connects the mature PR cluster to the retinal floor, since the cone cell plate is attached to the retinal basement membrane. Thus adherens junctions serve to maintain the integrity of the imaginal disc while also allowing the tissue to undergo gross morphological changes.

Shortly after this anchoring process at 55% pd, the entire retina dramatically increases in depth perpendicular to the plane of the epithelium, resulting in a barrel shaped PRC cluster. Adult retinas reach a final depth of about 100 μm, from 20 μm at 55% pd. It is not known what the exact forces that regulate this process are, although a report by Pellikka *et al* demonstrated that Crumbs (Crb), an apical polarity determinant with an extensively described role in regulating epithelial polarity, could play a role in this process. Pellikka *et al* showed that loss of Crb could lead to defects in rhabdomere extension, which resulted in short, bulky rhabdomeres at the apex of the ommatidia (Pellikka et al., 2002).

The final assembly of the ommatidia is as described in Fig. 1.5. The adult ommatidium is composed of 19 cells, 8 of which are PRCs, and 11 accessory cells. PRCs 1-6 are organised in a trapezoidal shape, the central space of which is occupied by PR7 and 8. PR1-6 extend from the pseudocone to the retinal floor, however, since R7 and R8 are positioned on top of each other, they extend to half the length of the other PRCs. Above and below this PRC chamber reside the cone cells and pigments cells, and the cone cell plate respectively.

In addition to this reiterating hexagonal array of 800 such ommatidial units throughout the eye, the adult eye also displays a chirality, whereby ommatidia, with their characteristic asymmetric and trapezoidal shape, 'point' towards the dorsal or ventral field, depending which side of the horizontal equator they are in. The dorsal and ventral fields are mirror images of each other. This chirality is set up soon after PRC recruitment and assembly of the ommatidial precluster, when the precluster begins to rotate 90 degrees, such that this mirror image of the dorsal and ventral halves are generated (Ready et al., 1976; Tomlinson, 1985; Wolff and Ready, 1991). This mode of organisation is termed planar cell polarity (PCP).

**Fig. 1.5 The *Drosophila* adult ommatidium.** (A) The adult ommatidium is composed of a stereotypical assembly of 19 cells. These include 8 photoreceptors cells, which are subdivided into 3 groups: R1-6 which lie in a circular pattern around the central 2 PRCs, R7, the distal or outer PRC, and R8, the proximal or inner PRC. A further 11 accessory cells make up the full complement of ommatidial cells. These include the cluster of 4 cone cells which overlie the PRC chamber, and form the lens floor. 2 primary pigment cells, secondary and tertiary pigment cells, and eye bristles arranged in a honeycomb configuration enclose the PRC chamber (from D Ready, 1993).

### *1.4.2.1 Cone cell pattern formation*

Cone cells are the lens secreting cells that form the floor of the simple lens. During the third instar, cone cells displace from below the developing proneural cluster, to take up a position above the PRCs. Shortly afterwards, cone cells form the characteristic cross-like arrangement of 4 cone cells seen in the adult ommatidium [Fig. 1.6]. At 14% pd, the anterior and posterior cone cells extend to the retinal basement floor to form part of the cone cell plate, and this extension separates the PRCs into 2 groups. A later extension by the equatorial and polar cone cells at 37% further subdivide the PRCs, as the processes now run between R1 and R7, and R3 and R4 respectively, and complete the cone cell plate at the base of the PRCs. These cone cell feet subsequently expand until 55% pd, when PRC apical surfaces become anchored to it through newly formed junctions (Longley and Ready, 1995).

Pattern formation of the cone cells is driven by the differential expression of cadherins. The arrangement of cone cells that is achieved through this mechanism is similar to that seen with a cluster of four soap bubbles [Fig. 1.6]. Soap bubbles will adopt a configuration that is thermodynamically stable, by minimising the surface area of the cluster. In a similar fashion, the configuration of cone cell clusters follows this principle, but is mediated through the expression patterns of cadherins and immunoglobulins (Bao and Cagan, 2005; Hayashi and Carthew, 2004).

*Roi* mutants have a variable number of cone cells per cluster, instead of the usual 4, but in all cases, the configuration of these groups could be predicted from the behaviour of soap bubbles with the analogous number of

bubbles. In contrast, the loss of appropriate cadherin expression in cone cells, as in *DE-cad* loss of function or gain of function mutants, could lead to the loss of this tendency to minimise the surface area of a group of cells; cone cells that have lost DN-cad expression increase the interface with the surrounding pigment cells and reduce the interface with other cone cells, demonstrating that appropriate cadherin expression was essential for cone cell pattern formation.

**Fig. 1.6 Cone cell movement and the elaboration of distinct PRC membrane domains during pupation.** (A) During the third instar, cone cells remain below the developing proneural cluster. Apical and basal domains are situated at the top and bottom of the epithelia respectively, and at this stage the epithelia is a simple columnar epithelium. Adherens junctions (z.a.) form an apicolaterally positioned belt which encircle the PRC, and separate the apical and basolateral domains. (B) At 37% pd, the cone cells have moved above the PRCs, and established new z.a. contacts with the PRCs below. The PRCs have inverted their apices to face the centre of the cluster, and the rhabdomere (wavy lines) begins to mature. The cone cell plate has also begun to form. (C) At 55% pd, all features of the adult ommatidia have matured, e.g. the inter-rhabdomeral space (IRS), the cone cell plate, and the apical domains of the PRCs have dramatically expanded. The elongated apical domains have also anchored to the cone cell plate, and the distal ends of the apical domain have anchored to the overlying cone cells. In addition, secondary and tertiary pigment cells have also been specified (basal lamina, b.l; cone cells, c.c; cone cell plate, c.c.p;focal adhesion, f.a; stress fibres, s.f; interhabdomeral space; IRS) (adapted from Longley *et al,* 1995).

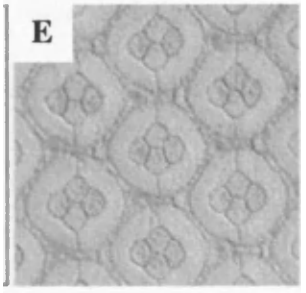



**Fig. 1.6 (cont) Cone cells.** (D) Sideview schematic of a Drosophila ommatidium at 35% of pupal life. Cone cells (eqc, equatorial; pc, posterior; plc, polar; ac, anterior) are surrounded by two primary pigment cells (pp) plus secondary pigment cell (sp), tertiary pigment cells (tp) and bristles (b). The cone cells sit over a cluster of photoreceptor cells (R) (adapted from Longley *et al,* 1995). (E) A retina stained with cobalt sulphide, illustrating the reiterating pattern of cone cell clusters. (F) A cone cell cluster with an arrow marking the junctional interface between the cone cells (adapted from Hayashi *et al,* 2004).

### *1.4.2.2 The maturation of the rhabdomere and the development of distinct membrane domains*

At approximately 37% pd, the apical surfaces of the PRCs begin to differentiate into rhabdomeres, the apical light-sensing organelle of the PRC. Most of the growth of the rhabdomeres occurs between 30-60%. The rhabdomere is assembled by the gradual addition of 60,000 microvilli stacks, which comprise 90% of the PRC membrane - these highly packed apical membranes contain high concentrations of the light sensing pigment. The rhabdomeres continue to form and elongate in this way to reach their adult form late in pupal development.

At approximately 50% pd, two distinct domains become apparent apical to the ZA; the apical domain, described above, and a stalk membrane domain, which is the region of the membrane that connects the rhabdomere to the ZA. Previously apical molecular markers become resolved to either the apical or subapical membranes, and the stalk membrane rapidly develops by recruiting new membrane.

The development of both these processes are regulated by Crb (Izaddoost et al., 2002; Pellikka et al., 2002). Two groups showed that two distinct domains of the Crb protein are responsible for independently mediating these processes. The intracellular portion of Crb is required for the integrity of the zonula adherens, to specify apical membranes and facilitate rhabdomere elongation, while the extracellular domain regulates the length of the stalk domain [Fig. 1.7].

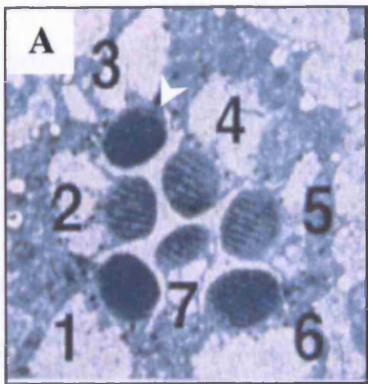



**Fig. 1.7 The *Drosophila* adult ommatidium.** (A) The adult rhabdomere (white arrowhead) is composed of a stack of microvilli. (B) Schematic of the adult ommatidium. The adult ommatidium is composed of 8 PRCs, assembled in a stereotypical trapezoidal arrangement (adapted from Pellikka *et al,* 2002). (C) PRCs in the third instar and during pupal life. The PRCs are elongated in the proximal-distal axis. This deepening of the epithelium occurs from 37% to adulthood, and the rhabdomere (blue) matures from 37% pd onwards (adapted from Izaddoost *et al*, 2002).

## 1.5 Epithelial polarity complexes in *Drosophila*

The imaginal eye disc begins as a simple columnar epithelium with clear apico-basal polarity, which subsequently undergoes significant morphogenetic changes in order to form the adult compound eye. The movement of the leading edge of differentiation, the MF, through the imaginal eye disc, signals the beginning of a shift in cell polarity – from the simple apical-basal polarity of the columnar epithelium, to the elaborate multidirectional polarity seen in the adult retina.

Complexes that have been shown to regulate and direct these processes include the Par complex (aPKC/Par-6/Bazooka) and the sub-apical region complex (Crumbs/Stardust/DPATJ) that specify the apical domain, and a basolateral complex (Lethal Giant Larvae, Discs Large and Scribble).

### 1.5.1 The Par complex: *aPKC/Par-6/Baz*

In order to generate cell polarity, cells segregate polarity determinants into different cellular domains. In *Drosophila*, Bazooka (Baz), aPKC, and Par-6 are members of the apically localised partition defective (Par) complex. Components of the Par complex have been shown to have central and early roles in the generation and maintenance of cell polarity, both in invertebrate and vertebrate systems.

The members of this complex are highly conserved, and the establishment of apicobasal polarity in diverse systems require the membrane

associated aPKC-Par-6-Baz complex. The asymmetric distribution of the Par complex was shown to be essential for the proper polarisation of *Drosophila* oocytes (Benton and St Johnston, 2003b; Vaccari and Ephrussi, 2002), the apical and basolateral membranes of *Drosophila* epithelial cells (Cox et al., 2001; Hutterer et al., 2004; Kuchinke et al., 1998; Shulman et al., 2000; Wodarz et al., 2000), and mammalian cultured epithelial cells (Bohm et al., 1997; Izumi et al., 1998; Suzuki et al., 2001) [Fig. 1.8].

The localisation of this complex in the *Drosophila* retina has been well characterised. In the imaginal eye disc and in early pupal development, aPKC and Par-6 localise apically, with Baz just basal and co-localising with Arm (Nam and Choi, 2003). aPKC and Par-6 can be resolved from other apical markers between 40 and 50% pd, as during this period both proteins localise to the stalk membrane domain, the membrane that connects the apical rhabdomere to the ZA. This pattern continues until approx 60% pd, after which aPKC, Par-6 and Baz migrate into the apical domain (Nam and Choi, 2003). Although physical interaction of Baz with aPKC and Par-6 has been demonstrated by co-immunoprecipitation experiments, the localization of Baz with AJ is consistent throughout early and mid pupal development and this was also shown to be the case in embryos (Harris and Peifer, 2004).

### *1.5.1.1 DaPKC/aPKC*

*Drosophila* atypical protein kinase C (DaPKC) is a key polarity determinant, regulating the localisation not only of the Par complex, but also other apical and basolateral complexes (Sotillos et al., 2004). DaPKC shows high sequence similarity to PKC-γ and PKC-ζ in mammals. In *Drosophila*,

DaPKC localises to the apical and sub apical membrane (Cox et al., 2001), and in mammalian cell lines, to the tight junctions (Izumi et al., 1998; Yamanaka et al., 2001).

Loss of function clones of *DaPKC* in the *Drosophila* pupal retina leads to polarity defects and the displacement of the junctional component Armadillo (Nam and Choi, 2003). Other studies conducted in *Drosophila* as well as other systems have demonstrated that aPKC regulates the position of both apical and basal determinants and promotes the development of complementary membrane domains by mutual antagonistic interactions. A number of studies demonstrated that the kinase activity of aPKC was necessary for these interactions (Hurov et al., 2004; Sotillos et al., 2004; Suzuki et al., 2003; Suzuki et al., 2004; Yamanaka et al., 2003).

One such polarity protein that is regulated in this way by DaPKC is a component of the basolateral complex, Lethal giant larvae (Lgl). *DaPKC* null mutants show reduced proliferation in neuroblast and epithelial cells, the opposite to that seen in *lgl* mutants, and in addition, reduced aPKC activity strongly suppresses most cell and overproliferation phenotypes seen in *lgl* mutants. Lgl also no longer localises apically in *aPKC* neuroblasts (Rolls et al., 2003).

Mammalian studies demonstrated that a mammalian complex of mPar-6C and aPKC could bind, phosphorylate and regulate mLgl *in vivo*, and Yamanaka *et al* also showed that mLgl could compete with PAR-3 (Baz homologue) for binding to and phosphorylation by aPKC and PAR-6 (Yamanaka et al., 2003).

A similar regulatory relationship exists between DaPKC and Crumbs (Crb), whereby phosphorylation of Crb by DaPKC functions to restrict Crb localisation to the apical domain. Moreover, the phenotype of overexpressed Crb could be alleviated by reducing DaPKC activity, demonstrating that DaPKC functions to regulate Crb activity and localisation (Sotillos et al., 2004).

Two studies on aPKC in mammalian cell culture showed that this mechanism was conserved in mammalian systems. Suzuki *et al*, and Hurov *et al* both showed that Par-1 localisation and activity could be regulated by phosphorylation in an analogous way to that observed for Lgl and Crb (Hurov et al., 2004; Suzuki et al., 2004), demonstrating that the function of DaPKC is mediated through conserved interactions and lateral exclusion mechanisms between DaPKC and polarity determinants in complementary domains.

### *1.5.1.2 Bazooka*

Baz is a multi-PDZ domain protein, which associates with DaPKC and Par-6. Baz functions as a scaffold protein, and in *Drosophila,* Baz binds directly to DaPKC (Wodarz et al., 2000), and is required for the establishment of apicobasal polarity in epithelia and neuroblasts.

*Baz* null embryos show defects in membrane polarity, and in the absence of Baz in pupal photoreceptors, the apical membrane domain fails to form, indicating that Baz is required for both early embryonic polarity, as well as morphogenetic events later in development (Hong et al., 2003b). Nam *et al* showed that in *baz* clones in the pupal retina, DPATJ, a member of the SAR complex was no longer detectable and Arm was mislocalised, demonstrating

that Baz is essential for the correct localisation of SAR complex proteins and the AJ component Arm (Nam and Choi, 2003).

The majority of studies examining Baz function in *Drosophila* tissues were conducted in the oocyte, and revealed that Baz is required for the early development of polarity during cellularisation in the *Drosophila* oocyte. Mutant analyses revealed that while Baz could localise apically in the absence of AJs, the assembly of junctional complexes required prior positioning of Baz, placing Baz as one of the earliest cues for epithelial polarity during cellularisation (Harris and Peifer, 2004). Baz, together with Arm and Sdt was shown to be upstream of ZA formation, since embryos mutant in these genes failed to form a ZA (Muller et al., 1996). These results were consistent with the localisation of Baz, since in embryonic epithelia Baz co-localizes with the sub-apical region (SAR) complex at the apical membrane and at the AJs, while in photoreceptors, Baz displays distinct localization from its complex members aPKC and Par-6, and is observed basal to this complex, at the AJs. In addition, the overexpression of the intracellular part of Crb in the eye could lead to the ectopic recruitment of both Baz and Arm, suggesting that Baz may be an integral component of the AJs (Nam and Choi, 2003).

Studies of the positioning and segregation of apical cues in the Drosophila embryo showed that aPKC and Par-6 are dependent on Baz for their apical localisation, and Baz itself is localised by cytoskeletal cues (Harris and Peifer, 2005). Wodarz *et al* showed that *baz* mutants could lead to loss of DaPKC localisation at the apical membrane. In contrast, overexpression of Baz could recruit DaPKC to ectopic lateral and basal positions in epithelia and neuroblasts (Wodarz et al., 2000). This is consistent with its domain structure

since Baz is a protein with three PDZ domains, and may function as a scaffold protein.

### *1.5.1.3 Par-6*

Par-6, also a PDZ domain protein, is apically recruited by Baz, and the overexpression of Baz leads to the formation of ectopic Baz-Par-6 complexes at the cortex. However, Par-6 is in turn required for the correct localisation of Baz, since in the absence of Par-6, Baz fails to localise apically in epithelial cells and neuroblasts, and instead redistributes to the cytoplasm, illustrating the mutual interdependence of these proteins for their localisation (Petronczki and Knoblich, 2001).

Petronczki *et al* showed that, as with its binding partners DaPKC and Baz, *Drosophila* Par-6 was required to maintain epithelial polarity, as well as direct normal asymmetric division in neuroblasts (Petronczki and Knoblich, 2001). Studies in the *Drosophila* oocyte revealed that Par-6 was additionally required for early anterior-posterior polarity, and oocyte fate (Huynh et al., 2001).

Nam *et al* revealed that Par-6 could directly bind to DPATJ, a member of the SAR complex, thus providing a mechanistic basis for the mutual dependence of the two complexes (Nam and Choi, 2003), and experiments in *Drosophila* embryos revealed a mutual antagonistic relationship between Par-6 and Lgl, a member of the basolateral polarity complex (Hutterer et al., 2004), demonstrating a role for Par-6 in the regulation of distinct polarity complexes.

### *1.5.1.4 Localisation mechanisms of the Par complex*

Once all components are apically localised in a sequential manner, the three proteins regulate each other to maintain their apical localisation (Macara, 2004; Nagai-Tamai et al., 2002; Ohno, 2001). Mutual interactions between aPKC and PAR-6 occur through their N-terminal PB1 domains (Suzuki et al., 2003; Suzuki et al., 2001). PAR-3, which is the mammalian homologue of Baz, is a scaffold protein, and was shown to interact with the kinase domain of aPKC through its central region, which is conserved in other species. Interestingly, the phosphorylation of this domain by aPKC decreases its affinity for aPKC (Izumi et al., 1998; Nagai-Tamai et al., 2002). This phosphorylation was shown to be crucial for the normal functioning of the Par complex, since mutation of the aPKC phosphorylation site in PAR-3 could impair the activity of the Par complex. Baz interacts dynamically with the Par-6-aPKC complex, and is often observed as slightly basal to aPKC and Par-6 (Harris and Peifer, 2005; Nam and Choi, 2003; Tabuse et al., 1998).

Since Baz is observed to localise independently at the apical membrane, through direction from cytoskeletal cues, it is likely to be involved in targeting the aPKC-Par6 complex to the appropriate membrane, however the subsequent maintenance of Baz as well as aPKC and Par6 is dependent on mutual interactions between the three components. In particular, the kinase activity of aPKC and the binding activity of PAR-6 to Cdc42 have been reported to be indispensable for the maintenance of Baz at the apical membrane (Hakeda-Suzuki et al., 2002; Hutterer et al., 2004).

#### *1.5.1.5 The Par complex regulates the localization of the sub-apical region complex*

As well as self-regulation of localisation, the Par complex is also reported to be necessary for the localisation of a second apical group, the Crumbs-Stardust (Sdt)-Pals1-associated TJ protein (Patj) complex, hereafter referred to as the sub-apical region (SAR) complex. A physical interaction between the 2 groups was demonstrated; Par-6 can bind to DPATJ (Nam and Choi, 2003) and DaPKC can bind Crb and DPATJ (Sotillos et al., 2004). In addition, localisation of the SAR complex by the Par complex is also mediated through aPKC phosphorylation of Crb. DaPKC binds to both Crb and DPatj, and the phenotype resulting from overexpressed Crb is suppressed by reducing DaPKC kinase activity, indicating regulation of Crb by DaPKC is necessary for both its localisation and activity (Sotillos et al., 2004). Kempkens *et al* also demonstrated that Par-6 could bind Crb *in vivo* (Kempkens et al., 2006), further demonstrating a mutual interdependence of these two complexes.

### *1.5.2* The SAR complex: *Crb/Sdt/DPATJ*

The SAR complex consists of three components: Crb, Sdt and DPATJ. In the developing pupal retina these proteins localise first to the apical domain, and later during mid pupal development, to the developing region immediately adjoining the apical region, the sub-apical membrane.

The SAR complex proteins act as positional cues for the appropriate formation of PRCs and adherens junctions in the eye, and loss of function

mutants of these proteins show severe disruption of polarity and morphogenetic defects in the retina.

Members of the SAR complex are highly conserved in vertebrates as CRB3, Pals1, and PATJ (Roh et al., 2003; Roh et al., 2002) [Fig. 1.8]. Many studies have demonstrated that the underlying interactions between the members of this complex, and its interaction with other polarity complexes are also conserved, and in mammalian epithelial cells, the CRB3-Pals1-Patj complex localises to the tight junctions, where it has a role in the establishment of polarity (Roh et al., 2003).

### *1.5.2.1 Crumbs*

Crb is a transmembrane protein with a conserved carboxy terminal PDZ binding motif that is used to bind to the PDZ domain proteins, Stardust (Sdt) and DPATJ. The gene was so named because the cuticle of Crb null embryos resembled breadcrumbs. This phenotype was shown to be the result of a loss of epithelial organisation, with a rounding of apoptotic epidermal cells that resembled breadcrumbs, and loss of function mutations in *crb* led to severely disorganised epithelia. Insertion of the Crb protein into the plasma membrane was shown to be sufficient to confer apical character (Knust et al., 1993; Tepass et al., 1990; Wodarz et al., 1993; Wodarz et al., 1995), demonstrating the functional role of Crb in the development of the apical domain, and overexpression of Crb could also lead to the expansion of the apical domain at the expense of the basolateral domain (Wodarz et al., 1995).

Crb is also critical for the correct positioning and formation of ZA, since ZA were unable to develop and coalesce into belt like structures in *crb* mutants (Grawe et al., 1996; Tepass, 1996).

Pellika *et al* examined the *crb* phenotype in the *Drosophila* pupal retina, and showed that in addition to defects in the ZAs, loss of Crb could lead to defects in the extension of the PRCs in the proximo-distal axis that occurs at approx 55% pd. *crb* mutant PRCs were short, and bulky, and did not extend to the retinal floor as in wild type retinas (Pellikka et al., 2002).

Two groups showed that distinct regions of Crb performed different functions in the maturation of the PRC, where the intracellular region of Crb was required for the proper formation of the ZA, apical membranes, and rhabdomeres elongation, while the extracellular domain was found to independently regulate the length of the stalk membrane (Izaddoost et al., 2002; Pellikka et al., 2002).

Crb function seems to be conserved in mouse and humans, since expression of the intracellular domain of mammalian CRB1 in *Drosophila* mutant for *crb* can rescue the cuticle phenotype (den Hollander et al., 2001).

### *1.5.2.2 Stardust*

Crb can bind the scaffold protein Sdt through its PDZ domains. Sdt is a single PDZ domain MAGUK (membrane-associated guanylate kinase) protein that was demonstrated to be important for the development of apical and subapical regions in the PRC. The mutant phenotypes of these two genes in the *Drosophila* eye are similar to each other, with a few distinct differences that indicate some disparity of function.

Loss of *sdt* strongly phenocopies the loss of *crb* phenotype, indicating that a primary role of Sdt may be to recruit Crb to the stalk membrane, and in addition, *sdt* was shown to be downstream of *crb* since the overexpression of Sdt could rescue some of the *crb* phenotype. However Sdt is also likely to be involved in the maintenance of rhabdomere integrity, since examination of the ultrastructure of rhabdomeres in *sdt* mutants revealed extensive degeneration and overextension of microvilli at the base membrane (Hong et al., 2003b).

Studies in *Drosophila* epithelial cells also revealed a role for Sdt in the polarised organisation of the epithelia, as well as a distinct role in the regulation of junction formation. In the epithelia, Sdt colocalises with Crb at the apicolateral cortex, but in *sdt* mutant embryos, Crb is mislocalised to the cytoplasm (Tepass and Knust, 1993), and the embryonic epithelia exhibits severe defects in apicobasal polarity, tissue organisation and cuticle formation (Bachmann et al., 2001; Hong et al., 2001; Muller and Wieschaus, 1996).

Together with Crb, Sdt was also shown to be essential in the biogenesis of AJs. Grawe *et al* first showed that ZAs failed to form in both *sdt* and *crb* mutant embryos (Grawe et al., 1996), and demonstrated that components of the ZAs, Armadillo and DE-Cad were also misdistributed in *sdt* embryos.

The mammalian orthologue, Pals1, similarly functions as an adaptor protein, acting as a linker for the mammalian orthologues of Crb and Patj (Roh et al., 2002), and knockdown experiments in MDCK cells revealed a conserved role for Pals1 in biogenesis of the AJs (Wang et al., 2007b).

### *1.5.2.3 DPATJ*

DPATJ, is also a multi-PDZ domain protein that localises to the apical cell membrane, in a complex with Crb, and Sdt. DPATJ is required to maintain the localisation of Sdt and Crb in the retina, and loss of *dpatj* in the retina leads to defects in stalk membrane biogenesis and rhabdomere morphogenesis. While loss of *dpatj* does not lead to the severe defects observed in *sdt* and *crb* mutants, DPATJ is essential for PRC morphogenesis during pupal development. In addition to the above functions, DPATJ also has a role in the positioning of the AJs (Nam and Choi, 2006; Richard et al., 2006).

Studies looking at the role of the mammalian homologue PATJ in epithelial morphogenesis have described a role for PATJ in stabilising the mammalian equivalent of the SAR complex, Crb3 complex, as well as a more generalised role in maintaining the spatial distribution of other polar components at the tight junctions (Michel et al., 2005; Shin et al., 2005).

**Fig. 1.8 Mammalian and invertebrate polarity complexes** (A) Polarised complexes, and their interactions in mammalian and invertebrate cells are closely conserved (from Nelson, 2003).

## 1.6 Par-1

Par-1, a serine-threonine kinase, is a polarity determinant with a conserved function in the generation of cell polarity. Par-1 localises to the posterior in the *Drosophila* oocyte (Shulman et al., 2000), and to the basolateral cortex in epithelial cells (Bayraktar et al., 2006). In *Drosophila*, *par-1* mutants show embryonic patterning defects, resulting from defects in the microtubule cytoskeleton (Shulman et al., 2000), and further studies showed that Par-1 was required for polarisation of the microtubule cytoskeleton in oocytes (Doerflinger et al., 2006). *Lkb1* mutants show similar defects in cytoskeletal organisation in the *Drosophila* oocyte, and a strong genetic interaction was observed between these 2 genes, suggesting that Par-1 and Lkb1 function in a conserved pathway to establish the anterior-posterior axis in *C.elegans* and *Drosophila* (Guo and Kemphues, 1995; Martin and St Johnston, 2003; Watts et al., 2000).

The Par-1 kinase was also shown to have a role in the development of cell polarity, and a role in the maintenance of cell polarity once established. In addition, loss of Par-1 could lead to the ectopic formation of AJs at the lateral membrane, indicating a further role for Par-1 in the regulation of AJ formation (Bayraktar et al., 2006).

In addition to cytoskeletal cues, the localisation of Par-1 is dependent on other polarity determinants. One such conserved interaction is the phosphorylation of human Par-1b (hPar-1b) by aPKC. aPKC acts upstream of hPar-1b to restrict its expression to the posterior cortex, by phosphorylating

hPar-1b on a conserved site. This phosphorylation negatively regulates the kinase activity, and prevents tethering of hPar-1b to the plasma membrane, thus restricting active hPar-1b to the lateral membrane (Hurov et al., 2004; Suzuki et al., 2004). Par-1 in turn regulates and restricts the movement of the apical Par complex by phosphorylating Baz and thus inhibiting the formation of a functional Par complex at the posterior cortex, where Par-1 is localised (Benton and St Johnston, 2003b), resulting in a lateral exclusion mechanism that is a common mode of action for many polarity proteins.

Previous work has revealed both genetic and cell biological evidence for an interaction between Lkb1 and Par-1, and most recent work, by Wang *et al* has shown that phosphorylation of Par-1 by Lkb1 is required for Par-1 activation in *Drosophila* (Wang et al., 2007a). Other work in *Drosophila,* by Martin *et al*, demonstrated that Lkb1 and Par-1 act in a common pathway to specify the A-P axis in the oocyte, and *in vitro* studies in mammalian systems identified LKB1 as an activating kinase for Par-1 (Brajenovic et al., 2004; Lizcano et al., 2004; Martin and St Johnston, 2003). Taken together, these studies suggest that Lkb1 has a conserved role in the regulation of Par-1 and Par-1 related processes.

## 1.7 Cancer and cellular polarity

Cancer has traditionally been thought as a disease of cell cycle control and regulation. As a result, tumour suppressors have classically been thought to have roles in control of cell cycle regulation, apoptosis, and other processes that affect growth. It is only recently that a more complex mode of action has

been suggested for tumour suppressors – in particular, dual role polarity determinants: e.g. Scrib, Lgl, Fat, etc., illustrate the intimate connection between polarity and growth control (Bilder et al., 2000).

Loss of polarity and changes in tissue architecture are often the hallmarks of oncogenic transformation in epithelial cancers. Many polarity determinants also have other functions in addition to their roles in polarity, which when deregulated affect other processes, e.g. aPKC and cell division (Rolls et al., 2003), Bazooka and motility (Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004), and Armadillo and cell proliferation (Morin, 1999). Further analysis of the mechanisms by which these polarity determinants regulate these distinct processes will serve to elaborate our understanding of the molecular basis of mammalian oncogenesis.

## 1.8 Aims of thesis

Previous work has demonstrated conserved roles for Lkb1 in the regulation of a number of different processes. The primary focus of these studies has been to characterise the functional roles of *Drosophila* Lkb1 in an *in vivo* context. I began by examining the *lkb1* loss of function phenotype in the adult retina. *Lkb1* clones present a pleiotropic phenotype, and the most striking defects, in PRC and rhabdomere morphology suggested that cellular polarity may be disturbed in *lkb1* clones. During the course of my studies, I sought to further characterise the mechanisms by which Lkb1 may regulate cell polarity and cellular morphology in epithelial tissue in *Drosophila*.

In the following chapters I will present and discuss evidence that the loss of *lkb1* results in severe defects in the expression patterns of key polarity determinants. Using the Flp/FRT system, I examined loss of function clones in the retina, and found that cells that lack *lkb1* frequently show a mislocalisation of polarity proteins e.g. Bazooka and aPKC; and that in the absence of *lkb1*, polarity markers from all domains were mislocalised.

In particular, I observed consistent localisation and accumulation defects in the junctional protein, Armadillo, and the polarity determinant Par-1, and genetic interaction assays suggest that the altered regulation of Arm and Par-1 may be contributing to the polarity defects observed in *lkb1* clones.

Defects in the localisation of junctional components Arm and DE-Cadherin prompted me to examine the morphology of ZAs in *lkb1* clones. I found that ZAs were also affected, occasionally appearing as fragmented junctions, were on average longer, and more numerous in individual PRCs, suggesting that in addition to the regulation of polarity determinants, Lkb1 may also have a role in the regulation of AJ formation.

In addition to the observed defects in PRC morphology, I also found severe defects in cone cell morphology and configuration. Since cone cell conformations are primarily regulated by adhesive forces, these results suggest further defects in adhesion in *lkb1* tissue and are consistent with a role for Lkb1 in regulating AJ formation.

Finally, in order to identify interactors of Lkb1, together with a colleague, Ruth Wheeler, I conducted a small-scale modifier screen of approximately 9,500 flies. Using the bi-partite GAL4-UAS system I obtained a modifiable rough eye phenotype that can be visibly enhanced or suppressed by

dominant mutations in interacting genes. Following rigorous tests, we recovered a number of mutants that are now ready to be mapped and identified.

# 2 Materials and Methods

## 2.1 Methods

### 2.1.1 Screen methods

#### *2.1.1.1 Isogenising screen stocks*

Mutations accumulate at a steady rate in a closed population such as fly stocks, and the presence of background lethals is likely. Thus for mapping purposes, as well as for consistent genetic interactions, it is desirable that the progenitor chromosome on which the mutations will be induced and the chromosomes which will be used for mapping are as identical as possible.

In *Drosophila,* this is achieved by the process of isogenising the chromosome to be mutagenised. We selected a third chromosome with the markers *pink peach* and *ebony* ($p\ ^{p}e$), and isogenised it by outcrossing single $p\ ^{p}e$ chromosomes to a balancer stock, and subsequently amplifying individual $p\ ^{p}e$ chromosomes by successive sib-matings. A lethal free stock $+;\ p\ ^{p}e$ line was established, and subsequently used as the strain to be mutagenised in the reported screens.

### *2.1.1.2 Mutagenesis*

Fly culture and crosses were performed according to standard protocols. The EMS protocol was adapted from Lewis and Bacher, 1968, as follows. 1-2 day old male flies isogenic for the second and third chromosomes of genotypes described in Fig. 3.4, were counted and desiccated by placing in empty bottles for 1 hr to ensure maximal uptake of ethyl methanesulfonate (EMS)/sucrose solution. A filter paper soaked in a 4-5mL of 25mM EMS/1% sucrose solution was placed in bottles and flies left to feed overnight. Males were subsequently transferred to fresh food bottles and left for 8hrs, before approx 40 EMS treated males were transferred to bottles containing 70-80 virgin females of the phenotypes described in Fig. 3.4. Flies were mated for 2 days at 29°C. Crosses were subbed every day for 3 days, and F1 progeny raised at 29°C. Males from the F1 generation were then examined for a modified rough eye or apterous phenotype.

Single male progeny with a dominant modifier effect, were mated to balancer genotype virgins to establish lines [Fig. 3.4]. These were subsequently retested against the original phenotype, and scored for the presence or absence of the modified phenotype. The ebony marker on the third chromosome was used to follow the mutagenised chromosome over the following crosses to establish lines, and to retest for modification of the phenotype. Lines that did not a reproducible modifier effect were discarded, and lines with a reproducible phenotype were next subject to further tests for false positives and specificity to Lkb1 as described in section 3.4.3

## 2.1.2 General Molecular Methods

### *2.1.2.1 DNA electrophoresis*

Agarose gels were prepared to a concentration of between 1-2% in TAE buffer. Ethidium bromide was added to the gel at a concentration of 0.5μg/mL and bands visualised on a UV transilluminator. Cloning were carried out as per standard procedures, using T4 DNA Ligase.

### *2.1.2.2 Cloning*

*pWiz[UAS-RNAi-Lkb1]*

This plasmid was generated by the amplification and cloning of a 250bp fragment (30-283bp) of Lkb1 into an RNAi vector (Lee and Carthew, 2003). This was cloned twice in opposite orientations in order to create a hairpin loop that will conditionally silence specific gene expression under the control of the UAS/Gal4 system [Appendix 1.1].

*Lkb1 fusion constructs for recombinant Lkb1 protein and antibody production*

The Lkb1 coding region was amplified and subcloned into pBAD/TOPO Thiofusion (Invitrogen), pGEX (GE Healthcare Life Sciences), and pMAL (New England Biolabs) vectors for bacterial protein expression in order to create tagged Lkb1 protein for recombinant protein and antibody production (Table 2-4) [Appendix 1.2].

### *2.1.2.3 Germline Transformation of pWiz[UAS-RNAi-Lkb1]*

Once the *p[UAS.RNAi-Lkb1]* construct was generated, an injection mix for microinjection of the construct into *Drosophila* embryos was prepared. The solution contained 10 µl of the *p[UAS.RNAi-Lkb1]* plasmid at 1µg/µl, and 9µl of the helper plasmid at 1µg/µl.

Subsequent steps were kindly carried out by the Drosophila Technical Unit. The construct was introduced into *yw Drosophila* embryos by microinjection (Fujioka et al., 2000), and hatching adults were back-crossed to *yw* individuals to establish lines and screen for transformants.

10 independent transformant lines were isolated, mapped to chromosomes and crossed to *GMR-Gal4* flies to screen for a phenotype.

### *2.1.2.4 Recombinant protein expression and purification*

Lkb1 fusion constructs (Table 2-4) were induced according to manufacturers instructions, and the construct with the maximal yield and purification was selected for recombinant protein production – pGEX-Lkb1.

A large-scale inoculation of pGEX-Lkb1 was prepared for expression and purification of Lkb1-GST. IPTG was added to an optimal final concentration of 0.5mM, and cells recovered by centrifugation after 4 hrs. Subsequent processing of the sample was carried out as per manufacturers instructions, including sonication of the cells, and solubilisation of the recombinant protein using Triton X-100. Purification was carried out using Glutathione Sepharose 4B and protein yield determined by measuring the absorbance at 280nm. The final concentration was 1.5mg/mL and was

processed for antibody generation by the Biological Resources Unit at Cancer Research UK.

### *2.1.2.5 Western analysis protocol*

Total protein levels were examined in 50% pupal development *lkb1* and wildtype retinas, as follows:

#### 2.1.2.5.1 Preparation of samples

3 pairs of retinas were dissected in cold PBS buffer, and transferred to PBS on ice. PBS was then aspirated and the samples immediately transferred onto dry ice. Dissection time was approximately 5 mins per 3 pairs of retinas. The samples were subsequently homogenised in 5µl 1x protein loading buffer.

#### 2.1.2.5.2 Protein Electrophoresis

Samples were adjusted for loading by Coomassie analysis. The samples were subsequently run on Invitrogen NuPage 4-12% Bis-Tris precast gels with MOPS running buffer. Approximately 4 retinas were loaded per lane.

#### 2.1.2.5.3 Western Blotting

Samples were transferred onto a nitrocellulose membrane as per standard blotting procedures in a wet transfer system. The filter was incubated in a 5% milk/PBS preparation at RT for 1 hr, and subsequent immunoblotting steps were carried out as standard, using antibody dilutions as described in

Table 2-2 and 2-3. Antibodies were diluted in 5% milk/PBS. Primary antibodies were incubated at 4°C overnight, and secondary antibodies incubated at RT for 2 hrs.

For the Odyssey Western Blotting system, gels and transfers were run as previously described. Odyssey blocking buffer/0.1% Tween-20 (0.4ml/cm3) was used in place of PBS-0.1% Tween-20, and the membrane protected from light during the secondary antibody incubation and last washes. A last wash of the membrane in PBS was also carried out to remove residual Tween-20 before scanning the membrane on the LI-COR Odyssey Infrared Imaging System.

## 2.1.3 Generation of mitotic clones in the eye disc and pupal retina

### *2.1.3.1 FLP/FRT clonal analysis*

Mosaic imaginal discs and pupal retinas were generated by crossing both *lkb1 4A4-2 82BFRT* and *lkb1 4A4-2 82BFRT* flies to the following stocks:

*eyFLP; 82BFRT w+*

*eyFLP; 82BFRT, P[mini-w] P[Ubi:nlsGFP], minute (M)*

*hsFLP; 82BFRT, P[mini-w] P[Ubi:nlsGFP]*

*hsFLP; 82BFRT, P[mini-w] P[Ubi:nlsGFP], M*

The Flp/FRT system was described by Golic *et al*, as a method to create mitotic clones in *Drosophila* tissues. Flp recombinase (flpase), when expressed in *Drosophila* can bind to transgenic Flp recombinase target (FRT) on identical

cytological sites on homologous chromosomes e.g. 82B, and induce site-specific recombination (Golic and Lindquist, 1989; Xu and Rubin, 1993). Regions of the chromosome that lie distal to the FRT site are made homozygous, and when cells divide, this leads to clones of either wildtype, heterozygous or mutant tissue. Clonal tissue created using this method can be detected by the absence of a marker that is present only on the wildtype FRT arm, such as green fluorescent protein (GFP).

The flpase is driven either by the *eyeless* promoter or the heat shock inducible *hsp70* promoter. The *eyeless* promoter constitutively drives expression of flpase in the eye antennal disc from the end of embryonic development.

The heat shock protocol was as follows: Egg lay: 24 hrs. Progeny were aged for 12 hrs. Heat shock was then performed for 2 hrs at 37°C, and after 22 hrs a second heat shock of 2 hrs was performed.

### *2.1.3.2 MARCM*

The MARCM system (Lee and Luo, 1999) was created to allow visualisation of mutant cells, but not the heterozygous or homozygous cells, and also allows the controlled expression of transgenes within mutant clones. The MARCM system utilises GAL80, a repressor of GAL4. MARCM flies contain a *GAL80* transgene, flanked by two homologous FRT sites. In heterozygous cells, GAL80 inhibits the expression of a GAL4 induced UAS-marker, in this instance, GFP. In homozygous mutant cells, the homozygous *GAL80* gene is recombined out by Flpase, and derepression of GAL4 results in the expression of a UAS-marker, and if desired, a UAS-transgene.

The MARCM system was used to create clones of *lkb1* marked by GFP, and expressing p35. *ey:FLP1 UAS:mCD8-GFP;; tub:GAL80 FRT82B tub-GAL4* flies were crossed to *UAS-p35; lkb1 FRT 82B 4B1-11/TM6B, Tb* flies and raised at 25°C.

Pupae were appropriately staged, and examined under the GFP microscope for GFP expression, and were subsequently dissected for immuno-histochemical analysis.

### *2.1.3.3 Gain of function clones*

The flip-out technique is used to generate gain of function clones in *Drosophila* tissue. Based on the Flp/FRT system, this method involves the use of Flpase and two FRT sites on the same chromosome to allow the constitutive expression of a gene of interest in patches of tissue. The genotype of the flip-out flies used in this assay is: *yw; Act5C<FRT y FRT>Gal4, UAS GFP.* The flpase, under a heat shock promoter, is induced in flies carrying the *flpase* and the flip-out transgenes, and the gene of interest. Activation of Flpase leads to the looping out of sequences between the two FRT sites, thus allowing the *Gal4* gene to be expressed, which subsequently leads to the expression of any transgenes under *UAS* control (Struhl and Basler, 1993).

This technique was used to create Arm gain of function clones.

## 2.1.4 Antibody staining of imaginal discs and pupal retinas

### *2.1.4.1 Selection of third instar larval discs and pupal retinas*

Wandering third instar larvae were selected for imaginal discs, and pupae were staged appropriately for pupal retinas; white prepupae (t=0) were aged at 20°C to the appropriate developmental stages (end of pupal development, t = 170hrs).

### *2.1.4.2 Dissection and fixation protocols*

Wandering third instar, or staged pupae were dissected in PBS or PB respectively, and fixed in PBS/4% formaldehyde or PB-F for 40 mins. Following this, three washes of 10 min are performed with PBS-Tx or PB-T respectively, and larval discs or pupal retinas are incubated in block buffer (PBS-B or PB-B) for 1 hr at RT.

### *2.1.4.3 Antibody staining protocol*

Imaginal discs or pupal retinas were incubated in primary antibody diluted appropriately (Table 2-2 and 2-3) in PBS-B or PB-B overnight at 4°C. Four washes of 10 min each were performed, and secondary antibodies (Table 2-3) added, diluted in the block buffers. Four washes of 10 min were performed, and discs and retinas were subsequently mounted on slides in Vectashield Mounting Medium (Vector Labs). Pupal retinas older than 40% were mounted using elevated coverslips, to prevent damage to the tissue. This was done using broken coverslips.

#### *2.1.4.4 BrdU staining protocol*

To measure and visualise cell proliferation, 5-bromo2'-deoxy-uridine (BrdU) can be incorporated into synthesising DNA in place of thymidine. Denaturation of the DNA is required to allow exposure to the antibody – this is achieved by acid treatment. BrdU is visualized following incorporation of BrdU using a monoclonal antibody against BrdU.

Discs were incubated in 75μg/mL BrdU in PBS for 40 mins. The discs are then fixed in PBS/4% PFA, and denatured in 3M HCl for 30 mins. Three washes of 10 min each were performed using PBS-Tx.

Antibody detection of BrdU is as described above.

#### *2.1.4.5 Confocal microscopy*

Confocal data was obtained on a Zeiss Axioplan Upright Laser Scanning Microscope using oil immersion lens and Zeiss LSM 510 software.

### 2.1.5 Histology of the compound retina

#### *2.1.5.1 Fixation and embedding of the retina*

The fixation protocol was the same for both adult retinas and pupal retinas, and for semithin (1μm) sections and transmission electron microscope ultrathin (70nm) sections.

Adult and pupal heads were decapitated and fixed for 30 min in equal volumes of 2% gluteraldehyde/0.1M NaPO4, pH 7.2 (SPB) and 2% osmium tetroxide/SPB. Following a wash with SPB, heads were transferred to 2% osmium/SPB for 1-2 hrs. An ethanol dehydration series of 30%, 50%, 70% and 90%, and 2 incubations of 100%, of 10 min each was carried out, followed by 2 10 min propylene oxide incubations. An equal volume of propylene oxide and Durcapan resin was added, and heads were incubated overnight at RT. A further incubation of 4 hrs at RT was carried out in pure resin, and samples were subsequently oriented in moulds and baked at 70°C overnight for sectioning.

### *2.1.5.2 Preparation of semithin and ultrathin resin sections*

Semi- and ultrathin sections were cut using a diamond knife and ultramicrotome. Semithin sections were dried onto slides, stained with Toluidine Blue, and mounted in mounting medium, DPX. Ultrathin sections were stained in uranyl acetate and transferred to copper grids.

### *2.1.5.3 Light microscopy and transmission electron microscopy*

Semithin sections were viewed on a Zeiss Axioplan microscope and images collected using OpenLab Modular Imaging software. Bright field images were viewed on a Nikon Eclipse E800 microscope and obtained using Nikon Act-1 Digital Imaging software. TEM samples were viewed using Jeol JEM 1010 and 1200 TEM microscopes.

#### *2.1.5.4 Scanning electron microscopy*

Adult flies prepared for SEM were immersed in 100% ethanol. Subsequent steps were carried out by the Electron Microscopy team at Cancer Research UK as described (Kimmel et al., 1990). Samples were analysed on a Jeol JSM 6700F Scanning electron microscope.

#### *2.1.5.5 Quantification of ZA lengths in ultrathin sections of mosaic pupal retinas*

To measure the length of ZAs in ultrathin sections of *lkb1* pupal retinas and wildtype pupal retinas, images of a number of sections from each genotype were viewed in Adobe Photoshop. The measure tool was used to take measurements of the individual ZA lengths.

Using the program GraphPad Prism 4, the data was then presented as a boxplot.

### 2.1.6 *In Vitro* Kinase Assay

#### *2.1.6.1 Production of recombinant proteins*

Bacterially expressed fusion proteins (Table 2-4) were purified according to manufacturer instructions and as described in section 2.1.2.4.

### *2.1.6.2 Kinase Assay protocol*

Recombinant LKB1/STRADα/mo25α protein, obtained from Upstate was used to assay for phosphorylation of candidate proteins by LKB1, using the following protocol:

Reaction mixture was prepared for each substrate containing 10µl of each of the following components: 1mg/ml solution of substrate in 5x Reaction buffer; Mg/ATP cocktail; and [$\gamma$-$^{32}$P] ATP].

These were dispensed into tubes containing 20µl of Lkb1/ STRADa/mo25a in 5x Reaction buffer, all kept on ice. The tubes were then transferred to a heated block set to 30°C. Termination of the assay was carried out at 2 time points, 30 min and 60 min, by adding SDS-sample buffer. The samples were subsequently run on a protein gel, and transferred as per normal western protocol.

Membranes were exposed to Kodak BioMax film at -70°C, overnight and autoradiographs developed using a film developer.

The kinase assay was repeated a second time to confirm the results. This time a digest was carried out on DPATJ-MBP using Factor Xa (New England Biolabs) as per instructions to cleave the MBP tag from DPATJ protein. 100 µg of Recombinant DPATJ-MBP was incubated with 3 µg of Factor Xa at RT overnight. The sample was subsequently run on a protein gel and analysed by Coomassie to ensure cleavage of the tag, and following this, 10 µg of the cleaved protein and tag were used in the kinase assay.

## 2.2 Materials

### 2.2.1 Buffers and solutions

Buffers and solutions used in DNA and protein electrophoresis, and other common molecular biology techniques are standard preparations.

*Immunofluoresence*

| | |
|---|---|
| PBS | Phosphate Buffered Saline |
| PBS-B | PBS, 5% Normal Goat Serum |
| PBS-Tx | PBS, 0.1-0.3% Triton-X |
| PB | 0.1M $NaPO_4$ pH 7.2 |
| PB-B | 0.1M $NaPO_4$ pH 7.2, 5% Normal Goat Serum |
| PB-Tx | 0.1M $NaPO_4$ pH 7.2, 0.1% Triton-X |

*In Vitro Kinase Assay*

| | |
|---|---|
| 5X Reaction Buffer: | 40mM MOPS-NaOH, pH 7.0,<br>1mM EDTA. |
| LKB1/STRAD/Mo25: | Dilute to 20-99.2ng/µl<br>with 20mM MOPS-NaOH, pH 7.0, 1mM EDTA,<br>0.01% Brij-35, 5% glycerol, 0.1% 2-<br>mercaptoethanol, 1mg/ml BSA. |
| Mg/ATP cocktail: | 100mM non-radioactive ATP and 75mM<br>magnesium chloride in reaction buffer |
| [gamma-32P]ATP: | 1 microCi/microlitre in Mg/ATP cocktail |

Substrate: 1mg/ml solution using 5x Reaction buffer

## 2.2.2 Fly Stocks and fly husbandry

Stocks are maintained at 20°C and all crosses carried out at 25°C unless otherwise stated. Flies are raised in bottles or vials containing standard fly media comprised of yeast, molasses and cornmeal.

| Fly Stock | Source/Notes |
|---|---|
| *lkb1 4A4-2 sr e ca /TM6B, Tb* | Martin *et a,* 2003; Deletion of M1 to Y145. Lethal. EMS mutant |
| *lkb1 4B1-11/TM6B, Tb* | Martin *et al,* 2003; amino acid replacement: Q98. Lethal. EMS mutant |
| *K147M.Scer/UAS.P/T.T:Avic/GFP/Cyo* | Martin *et al,* 2003; in vitro construct – site directed mutagenesis. Kinase dead Lkb1 |
| *S535A.Scer/UAS.P/T.T:avic/GFP/TM6B* | Martin *et al*, 2003; in vitro construct – site directed mutagenesis |
| *S535E.Scer/UAS.P/T.T:Avic/GFP/Cyo* | Martin *et al*, 2003; in vitro construct – site directed mutagenesis. Activated Lkb1 |
| *Scer/UAS.P/T.T:Avic/GFP/TM6B* | Martin *et al*, 2003; in vitro construct – coding region fusion. Wildtype Lkb1 |
| *p[genomic-Lkb1-GFP]* | Martin *et al,* 2003 |
| *pWIZ[UAS.RNAi-Lkb1]* | This study, Appendix 1.1, R. Carthew Lab |
| *yw, eyflp;82BFRT w+* | H. McNeill lab |
| *yw, eyflp;82BRT nlsGFP, M/TM6B, Tb* | This study |
| *yw, hsflp;82BFRT nlsGFP* | H. McNeill lab |
| *yw, hsflp;82BFRT nlsGFP, M/TM6b, Tb* | S. Leavers Lab |
| *Pink peach, ebony* | H. McNeill lab |
| *P{ry[+t7.2]=neoFRT}82B cu[1] sr[1] e[s] ca[1]* | BS 5748 |
| *Par-1[W3]/Cyo* | D. St. Johnston Lab. P element excision. |

| | |
|---|---|
| | Also removes mei-W68 transcription region |
| *p[UASp-par-1.N1S.GFP]* | D. St. Johnston Lab |
| *UAS-p35* | H. McNeill Lab |
| *y[1] arm[4] w[*]/FM7c, P{ry[+t7.2]=ftz/lacC}YH1* | BS 8554. Loss of function, amorph |
| *arm[2] P{w[+mW.hs]=FRT(w[hs])}101/FM7a* | BS 619. Loss of function, hypomorph |
| *hsflp; P[UAS $Arm^{WT}$]* | BS 7409 |
| *Flip out. Yw;Flp Act5C<FRT y FRT>Gal4, UAS GFP* | H. McNeill lab |
| *yw; P[neoFRT]82B p[tubP-GAL80]LL3 BS 5135* | H. Richardson lab |
| *Canton-S-SNPiso3* | BS 6366 |

**Table 2-1 Fly stocks**

### 2.2.3 Antibodies used in immuno-fluorescence and immuno-blotting experiments

| Antibody | Species | Dilution | Source |
|---|---|---|---|
| Armadillo (N Term) | Ms | 1:100 | DSHB |
| Armadillo (C Term) | Rb | 1:250 | Biogenes |
| Crb | Ms | 1:100 | DSHB |
| Crb (extracellular dm) | Rt | 1:250 | E. Knust Lab |
| Sdt | Rb | 1:100-200 | Yang *et al*, 2001 |
| PATJ | Rb | 1:2000 | M Bhat Lab |
| aPKC (c-20) | Rb | 1:400 | Santa Cruz |
| p-PKZ zeta T Loop (PPA-206) | Rb | 1:1000 | P.Parker Lab |
| Bazooka (N-term) | Rt | 1:500-1000 | A.Wodarz Lab |
| Par-6 | Rb | 1:500 | D.Montell Lab |
| DE-Cadherin | Rt | 1:100 | DSHB |
| DN-Cadherin (DN-Ex #8) | Rt | 1:100 | DSHB |
| D-Catenin (alpha-catenin) | Rt | 1:100 | Oda *et al*, 1993 |
| BarR1 | Rt | 1:500 | H.McNeill Lab |
| Prospero | Ms | 1:50 | DSHB |
| Elav | Ms, Rt | 1:20; 1:1000 | DSHB |
| Rough | Ms | 1:100 | DSHB |
| Spalt | Rb | 1:500 | Barrio *et al.*, 1999 |
| Boss | Ms | 1:1000 | Van Vactor *et al.*, 1991 |
| BrdU | Ms | 1:20 | Pharmingen |
| Caspase-3 | Rb | 1:200 | Cell Signaling Tech |
| Par-1 | Rb | 1:500 | Tomancak *et al*, 2000 |
| p-Par1 | Rb | 1:1000 | Ohno *et al*, 2004 |
| Dsh | Rt | 1:1000 | Shimada *et al*, |

| | | | |
|---|---|---|---|
| | | | 2001 |
| Wingless | Ms | 1:100 | DSHB |
| GSK3 | Ms | 1:100 | Upstate |
| p-GSK3 | Rb | 1:50 | Upstate |
| p-Beta-catenin (ser33/ser37/thr41) | Ms | 1:20 | Cell Signalling Tech |
| Beta-tubulin | Ms | 1:100 | DSHB |
| Na+/K+-ATPase (a5) | Ms | 1:50 | DSHB |
| GFP | Rb | 1:1000 | Molecular Probes |
| dLkb1 | Rt | 1:1000 | This study |
| dLkb1 | Rb | 1:250-500 | J. Knoblich |

**Table 2-2 Primary antibodies**

| Secondary | Dilution | Source |
|---|---|---|
| α-animal-cy3, cy5 and FITC | 1:300 | Jackson Immuno-Research Laboratories |
| α-animal-HRP (westerns) | 1:1000 | Jacskson Immuno-Research Laboratories |
| α-animal IR800, IR700 | 1:2000-10,000 | Rockland, Tebu-Bio |

**Table 2-3 Secondary antibodies**

## 2.2.4 Recombinant protein constructs

| Lkb1 construct | Vector Source | Lkb1 sequence |
|---|---|---|
| pWiz-UAS>RNAi-Lkb1 | R. Carthew Lab | 250bp |
| pBAD/TOPO-Lkb1 | Invitrogen | Full length |
| pGEX-Lkb1 | GE Healthcare Life Sciences | Full length |
| pMAL-c2X--Lkb1 | New England Biolabs | Full length |

**Table 2-4 Lkb1 constructs**

| Construct | Protein | Source | Vector Source |
|---|---|---|---|

| | | | |
|---|---|---|---|
| pGEX4T1-Baz(minus PDZ domain) | Baz-GST | K. Choi Lab | Amersham Biosciences |
| pGEX4T1-aPKC | aPKC-GST | K.Choi Lab | Amersham Biosciences |
| pGEX4T1-Par-6 | Par-6-GST | K. Choi Lab | Amersham Biosciences |
| pMal-CRI-Par-6 | Par-6-MBP | K. Choi Lab | Amersham Biosciences |
| pMal-Dlt | Patj (Dlt)-MBP | K. Choi Lab | New England Biolabs |
| pGEX-Crbi | Intracellular Crb | Y.N. Jan Lab | Amersham Biosciences |

**Table 2-5 Recombinant protein constructs used in kinase assay**

# 3 A genetic screen to identify interactors of Lkb1

## 3.1 Introduction

*Drosophila Melanogaster* has been a principal player of eukaryotic genetics for almost a century, and one reason for this is the relative diversity of genetic tools with which to manipulate the fly genome. In particular, forward screens whereby the phenotype or genetic outputs are used to identify genes that are involved in specified processes have been very successful in characterising many biological pathways (Nusslein-Volhard and Roth, 1989).

Due to the diverse methods available for generating genome wide mutations, screens of all sizes can be readily carried out – from small scale screens of just a few thousand flies, to saturating screens of tens of thousands of flies, that carry the promise of multiple alleles, complementation groups and the prospect of completely characterising a given process. The most efficient way to induce mutations remains the EMS method, described by Lewis and Bacher in 1968. This method induces a high frequency of point mutations in the genome with relative unbias, at a frequency of ~1 in 1000 genes, although larger genes accumulate more mutations than smaller genes, by virtue of their size. In general, screens utilising a concentration of 25mM EMS are expected to yield one hit per autosome on average, and one hit in 2000 to 5000 for most loci.

One drawback to mutagenesis by EMS however is the generation of mosaic progeny. If the germline is not mutant, the mutation will not be carried through to the next generation. Hence, F1 progeny must be backcrossed to the original phenotype to ensure that the mutation is heritable.

This chapter describes the characterisation of Lkb1 misexpression constructs for an EMS modifier screen to identify interactors of Lkb1, the primary screens performed, counterscreens and complementation analysis of recovered mutants, and the mapping strategy currently being utilised to map identified mutants. This work was carried out in conjunction with a member of the lab, Ruth Wheeler.

## 3.2 Characterisation of Lkb1 misexpression constructs

### 3.2.1 Lkb1 misexpression phenotypes in the eye

In order to carry out a modifier screen, it is necessary to produce a fly with a modifiable phenotype. Since the aim of this screen is to identify biologically relevant targets and interactors of Lkb1, I wanted to conduct the screen in a biological environment where the molecule is known to have a function, and where its natural substrates and effectors will be present. Lkb1 protein is expressed throughout the developing eye disc (chapter 4) and my initial studies examining *lkb1* loss of function clones in the *Drosophila* eye confirmed that *Drosophila* Lkb1 has a function in the eye. Loss of *lkb1* has a pleiotropic phenotype in the retina [described further in chapter 4 and [Fig.

4.2], demonstrating that therefore the eye would be an appropriate system in which to uncover targets of Lkb1.

Since loss of function alleles of *lkb1* are lethal, and carrying out a clonal assay screen would be unfeasible in terms of the work involved in identifying interactors, we decided to conduct a misexpression screen.

I carried out misexpression studies to characterise the phenotype of a number of *UAS-Lkb1* constructs. Using the bipartite misexpression system in flies called the *GAL4-UAS* system (Gustafson and Boulianne, 1996), I tested a number of constructs in flies for a phenotype that was scorable, consistent, and of moderate severity, which would enable both mutations that enhance and suppress the phenotype to be identified. I tested several different eye specific drivers *(Glass Multiple Reporter (GMR)-Gal4*), as well as 2 wing drivers (*MS10-* and *Apterous-Gal4*). *GMR-Gal4* drives high level expression of UAS constructs in the eye, posterior to the morphogenetic furrow. The wing drivers, *MS1096-Gal4* (BS 8696) and *Apterous-Gal4* (BS 3041) express Gal4 in the dorsal wing disc and in the Apterous pattern. The *UAS-Lkb1* constructs examined were:

*i.* *Wildtype Lkb1.*

*ii.* *Kinase dead Lkb1.* A transgene with an amino acid replacement K174M – shows no rescuing activity in *lkb1*germline clones.

*iii.* *Activated and non-phosphorylatable Lkb1. Drosophila* Lkb1 has a conserved phosphorylation motif on its C terminus, which in mammalian cells is phosphorylated by PKA. This phosphorylation was shown to be important in suppressing cell growth. 2 transgenes were generated, 1. Serine 535 is mutated to

alanine (S535A) to prevent phosphorylation, and 2. Serine 535 is mutated to glutamic acid (S535E) to mimic the presence of a phosphate group.

*iv.* *RNAi Lkb1.* A 250bp segment of dLKB1 was cloned into a *pWIZ-UAS* vector (see Materials and Methods).

The *UAS- Lkb1* constructs detailed in *i-iii* were previously characterised in Martin *et al,* which showed that all these constructs save the kinase dead construct were able to rescue Lkb1 function when overexpressed. In addition, when overexpressed, *UAS- Lkb1.WT* and *UAS- Lkb1.S535E* were able to rescue the defects in localisation of the posterior mRNA Staufen, as well as defects in polarity in follicle cells mutant for *LKB1. UAS-Lkb1.S535A* showed rescue of the lethality phenotype, but does not rescue Staufen localisation unless ten-fold overexpressed, and in addition, this construct could not rescue polarity defects in mutant follicle cells.

Thus these results demonstrate that these transgenes are able to produce functional Lkb1 that, to a greater or lesser degree, is able to substitute for endogenous Lkb1 function (Martin and St Johnston, 2003).

*UAS- Lkb1* constructs that result in both loss of function (Lkb1-kinase dead, -S535A, and -RNAi constructs) and gain of function (Lkb1-wildtype and activated), result in similar phenotypes, since both disrupt normal eye morphogenesis.

At 25°C, *GMR-Gal4* expressed alone result in a very subtle eye phenotype, with very occasional irregularities in ommatidial organisation. When these constructs are expressed using *GMR-Gal4* at 25°C, there is no

detectable difference in eye morphology from that observed with *GMR-Gal4* alone.

At 29°C, GAL4 is expressed at a higher level, and the *GMR-Gal4* eye is moderately rough, with a higher frequency of defects in ommatidial organisation, and occasional fusions of ommatidia [Fig. 3.1]. The Lkb1 phenotypes were tested at 29°C, and displayed eyes with more severe phenotypes than that displayed by *GMR-Gal4* alone. The phenotypes ranged from a moderate roughening of the ommatidial array e.g. *UAS- Lkb1.S535A*, to a moderate severe phenotype e.g. with *UAS-Lkb1.RNAi.*

The kinase dead, activated and RNAi Lkb1 constructs resulted in the most severe overexpression phenotypes, with fusion of ommatidia, resulting in a glassy surface of the eye, and loss of pigment in the dorsal half of the eye. The overexpression of these three constructs also often resulted in a protruding area in the dorsal part of the eye, with the *RNAi-LKB1* construct showing a protruding surface over the entire eye – similar to that seen in pinhead screens (Oldham et al., 2002) [Fig. 3.1]. These phenotypes are all dose-dependent, and increase in severity when two copies of the construct are present.

### *3.2.1.1 Microscopic examination of Lkb1 misexpression phenotypes*

Microscopic inspection of the *GMR-Gal4* driven phenotypes in semithin (1μm) resin sections of the eye revealed that these constructs also share similar phenotypes, such as a loss of photoreceptors cells (PRCs), and disruption of ommatidial patterning. *GMR-Gal4* expressed alone results in a variation in the size of the rhabdomeres (the apical projection of the PRCs). In addition, PRCs are often missing from the ommatidia, and the normal

stereotypical organisation of the PRCs in a trapezoidal pattern is sometimes disturbed. The *UAS- Lkb1* constructs displayed similar phenotypes and defects in semithin sections. *GMR- Lkb1.WT* and *GMR- Lkb1.S535A* also exhibited an apparent increase in pigment granules cells [Fig. 3.2]. Interestingly, I found that the overexpression of the *UAS-Lkb1.RNAi* construct in the adult eye, in addition to defects such as apoptosis and disorganisation of the ommatidial array, also led to deformed, bulky and fused rhabdomeres. This phenotype is also observed in loss of function *lkb1* clones in the imaginal eye disc, the pupal retina and the adult eye, and is examined more closely in the next chapter.

### 3.2.2 Lkb1 misexpression phenotypes in the wing

I next examined the overexpression phenotype of Lkb1 in the wing using *apterous-Gal4 (ap-GAL4)*, and *MS1096-Gal4*, and found that this led to strong, scorable phenotypes.

*Ap-GAL4* phenotypes are most obvious in the wing, the scutellum and macrochaetae bristles on the notum and scutellum. At 25°C and 29°C, expression of *ap-GAL4* alone leads to mild defects in the wing – the wings appear to be slightly raised from the body, and macrochaete bristles are no longer pointed in the same direction, often appearing misoriented [Fig. 3.1].

The *apGAL4- Lkb1* phenotypes at 29°C included a dichaete wing phenotype whereby the wings point outwards from the body and appear fixed at a 180° angle, a deformed and reduced scutellum and missing or misshapen scutellar and notum macrochaete. These defects in the wing and scutellum were apparent both at 25°C and 29°C for all Lkb1 constructs, but were more

pronounced at 29°C, with the dichaete wing and reduced scutellum phenotypes fully penetrant for the wildtype-Lkb1 and activated-Lkb1 constructs [Fig. 3.1].

Since the overexpression of activated-Lkb1 resulted in the strongest and most consistent phenotype, both in the eye and the wing, this construct was selected for the first trial screen of approximately 1000 mutagenised flies.

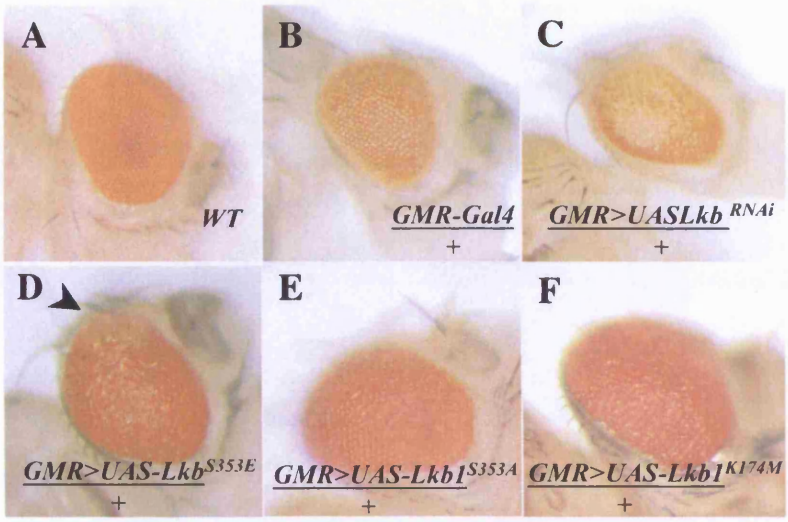



**Fig. 3-1 The misexpression phenotypes of Lkb1.** All phenotypes are at 29°C (A) Wildtype retinas display a smooth regular pattern (B) Eyes expressing *GMR-Gal4* display a mild rough eye phenotype, with perturbation of the regular ommatidial array. (C-F) Flies expressing (C) *GMR>Lkb1RNAi* (D) *GMR>Lkb1* activated (E) *GMR>Lkb1.S535A* (F) *GMR>Lkb1* kinase dead display more severe rough eye phenotypes. Flies overexpressing Lkb1 sometimes display loss of pigment, over the whole eye or at the dorsal half of the eye (black arrowhead; D). Ommatidia are frequently fused, resulting in a 'glassy' surface to eye.


**Fig. 3-1 (cont) The misexpression phenotype of Lkb1 constructs** All phenotypes are at 29°C (G) Wildtype scutellum (black arrowheads) and macrochaete. Macrochaetes on the notum and scutellum (white arrowheads) are stereotypical in placement, number, and orientation (from Kanuka *et al*, 2005) (H) Flies expressing apterous-Gal4 display missing macrochaete bristles at the notum and the scutellum (black arrow) and slightly raised wings (I-L) Flies expressing (I) *GMR>Lkb1.S535A* (J) *GMR>Lkb1 kinase dead* (K) *GMR>Lkb1WT* (L) *GMR>Lkb1 activated.* Flies overexpressing Lkb1 often display a deformed scutellum (I, black arrowhead), missing macrochatae at the scutellum, and a dichaete wing phenotype (K and L, black arrows).

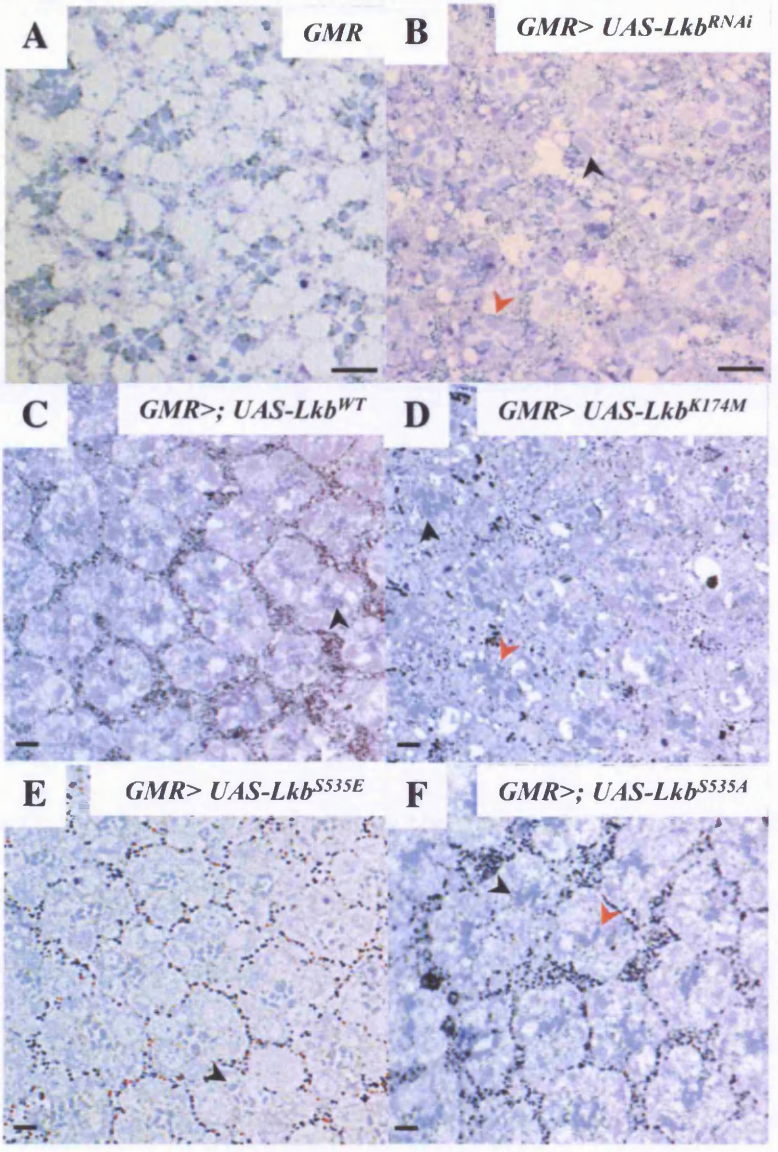



**Fig. 3-2 The misexpression phenotypes of Lkb1** Semi-thin tangential sections (1μm) through retinas overexpressing Lkb1 constructs (A) A section of a fly eye expressing *GMR-Gal4* alone. The phenotype includes photoreceptor cell (PRC) death, defects in PRC arrangement, and abnormal morphology of rhabdomeres. (B-F) Sections of fly eyes overexpressing Lkb1 constructs – ommatidia are frequently missing PRCs (red arrowheads), rhabdomeres are often bulky, deformed, or fused (black arrowheads), and PRCs are often not organised in the stereotypical trapezoid shape. Sections of eyes overexpressing *GMR>Lkb1WT* and *GMR>Lkb1.S535A* also reveal an apparent increase in pigment cells. These phenotypes are more severe in retinas overexpressing *GMR>Lkb1* constructs than in retinas expressing *GMR-Gal4* alone. Scale bars represent 10μm.

## 3.3 Testing for dominant modifications of the Lkb1 phenotype

As well as identifying a scorable phenotype, I also needed to obtain a phenotype that can be altered by dominant modifiers. Since the phenotype is generated by the overexpression of Lkb1, it is desirable that the rough eye phenotype is caused by the disruption of Lkb1 specific processes in the eye, rather than random interactions as a result of overexpression of a kinase. This would sensitise the assay to mutations in genes that are specifically involved in Lkb1 regulating or related processes, since reducing the gene dosage of these proteins by 50% will further impair the same processes and pathways that are affected by the overexpression of Lkb1.

Since the rough eye produced by the overexpression of kinase dead Lkb1 should be a result of a reduction in Lkb1 function, reducing the gene dosage of endogenous Lkb1 by 50% in eyes using the null allele *lkb1 4B1-11* with overexpressed kinase dead Lkb1 should exacerbate the rough eye phenotype. In fact, removing one copy of *lkb1 4B1-11* suppressed the phenotype; the eyes were smoother, possessed fewer ommatidial fusions and pigment was fully restored [Fig. 3.3].

I then overexpressed activated Lkb1 in a background heteroallelic for *Lkb1*, again using the null allelle *4B1-11* [Fig. 3.3]. Both in the eye and in the wing, the Lkb1 overexpression phenotype was suppressed, indicating that the overexpression phenotype of Lkb1 in both systems are likely to be due to the deregulation of Lkb1 specific processes. This result further demonstrated that

Lkb1 misexpression phenotypes are sensitive to a reduction in the dosage of genes that are involved in these processes, and can lead to a visible and consistent modification of the misexpression phenotypes.

Martin *et al* had also reported a genetic interaction of Lkb1 with Par-1 in the *Drosophila* oocyte; therefore it was pertinent to test for a modification of the phenotype by a reduction in Par-1 (Martin and St Johnston, 2003). Using the allele *par-1 W3* to reduce Par-1 levels in flies overexpressing activated Lkb1, I observed a consistent suppression of the phenotype [Fig. 3.3]. These flies had fewer ommatidial fusions, and no loss of pigment in the dorsal half of the eye.

Although reducing endogenous Lkb1 in flies with overexpressed kinase dead Lkb1 should have logically led to an enhancement of the phenotype, the interaction was specific, since in contrast, reducing the amount of the unrelated gene MAP205, a microtubule associated protein, did not lead to a modification of the phenotype (data not shown).

Taken together, these results provided evidence that the activated and kinase dead Lkb1 misexpression phenotypes were Lkb1 specific, and could be utilised in a dominant modifier screen to identify additional interactors of Lkb1.

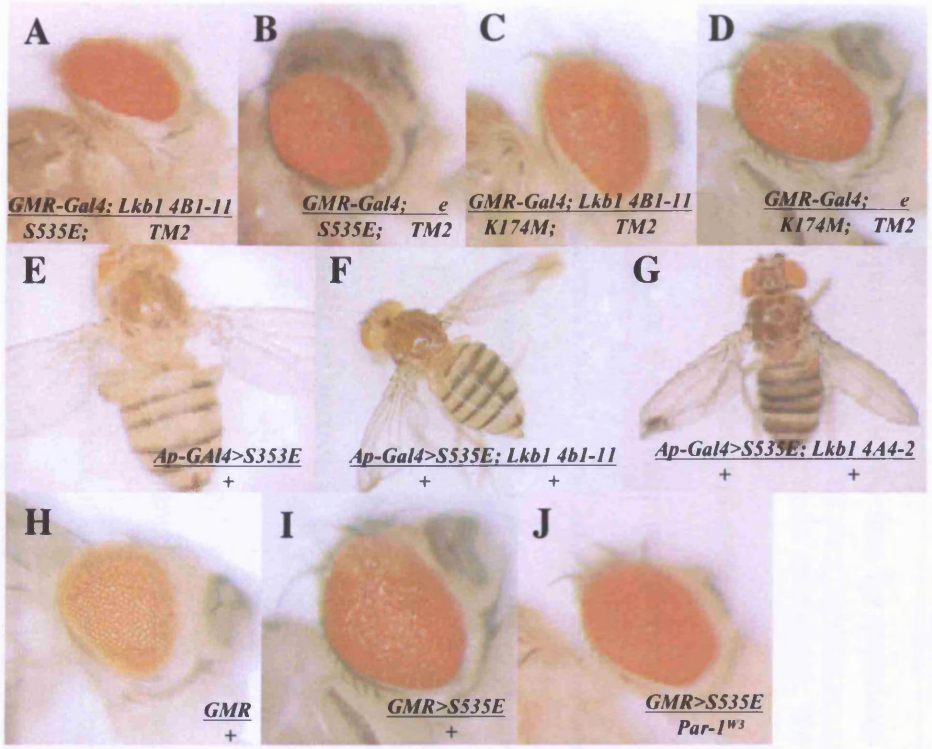



**Fig. 3-3 Reducing endogenous Lkb1 can modify the *lkb1* misexpression phenotype.** (A-D) The rough eye phenotype can be suppressed in flies expressing GMR>activated Lkb1 (A) or GMR>kinase dead Lkb1 (C) by removing one copy of the *lkb1* gene. (B and D) are controls. (E-G) Reducing endogenous Lkb1 in flies expressing apGal4-activated Lkb1 results in a subtle suppression of the overexpression phenotype. (H-J) Reducing endogenous Par-1, a reported Lkb1 interactor, results in a suppression of the GMR>activated Lkb1 rough eye phenotype (J). (H) is a control fly expressing GMR-Gal4 alone.

## 3.4 Screens

We performed the screen as described in Materials and Methods (section 1.1.1). We performed 3 pilot screens and 1 medium scale screen using different *UAS- Lkb1* constructs, as detailed in Figure 3.1 and Table 3-1. In total, we screened approximately 9,500 flies for mutations on the third chromosome that modified the Lkb1 misexpression phenotype. We screened for both enhancers and suppressors of the tester phenotype, of which 151 were recovered. Following retests, the total number of mutants obtained was 26, 6 of which were enhancers (*E(Lkb1)*, and 20 were suppressors (*S(Lkb1)*. The percentage of modifiers identified was 0.2-0.7% of the total number screened.

### 3.4.1 Genetic schemes

We selected and maintained robust mutants on the third chromosome. Genetic schemes for the identification, verification, and recovery of these mutants are described in Fig. 3.4. Since EMS mutagenesis can lead to the production of mosaic progeny, we also retested for the heritability of the phenotype in the next generation, to determine whether the mutation could be maintained as a stock.

Retesting the mutations for specificity to Lkb1 was also necessary before mapping to identify false positives; this includes mutants that interact with Gal4 itself, rather than the misexpression of Lkb1, as well as mutations in

house keeping genes that may affect the processing of Lkb1 rather than any Lkb1 specific pathways. Genetic schemes for these retests are as described in Fig. 3.4.

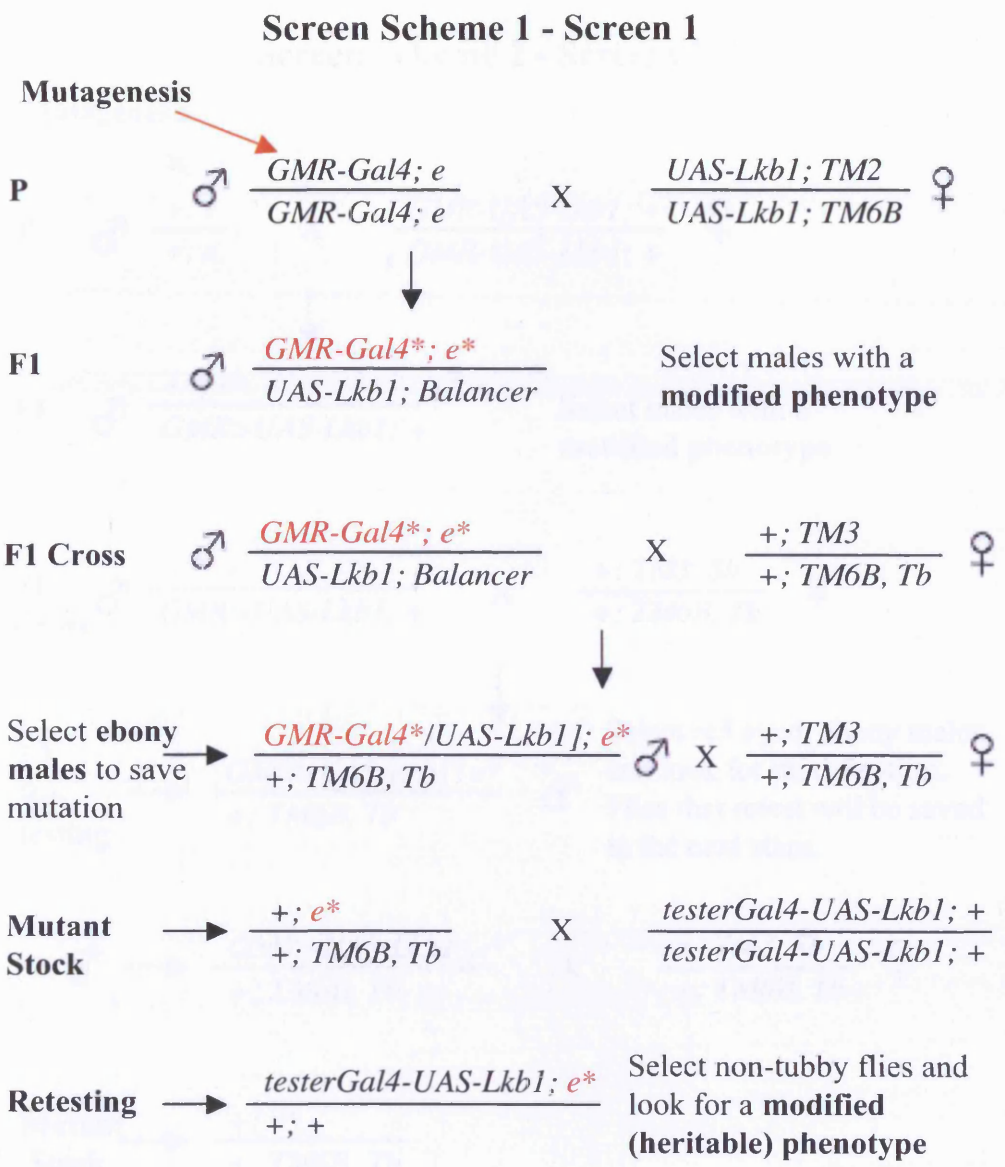



**Fig. 3-4 Genetic schemes for the primary Lkb1 screen** (A) Screen scheme for Screen 1. Females were crossed to mutagenised male stocks as indicated in the scheme. Male progeny were assayed for the ability to modify the tester phenotype, and lines were established by crossing to balancer stocks. At the same time, modifier lines were tested for mosaicism, by recrossing to the original phenotype.

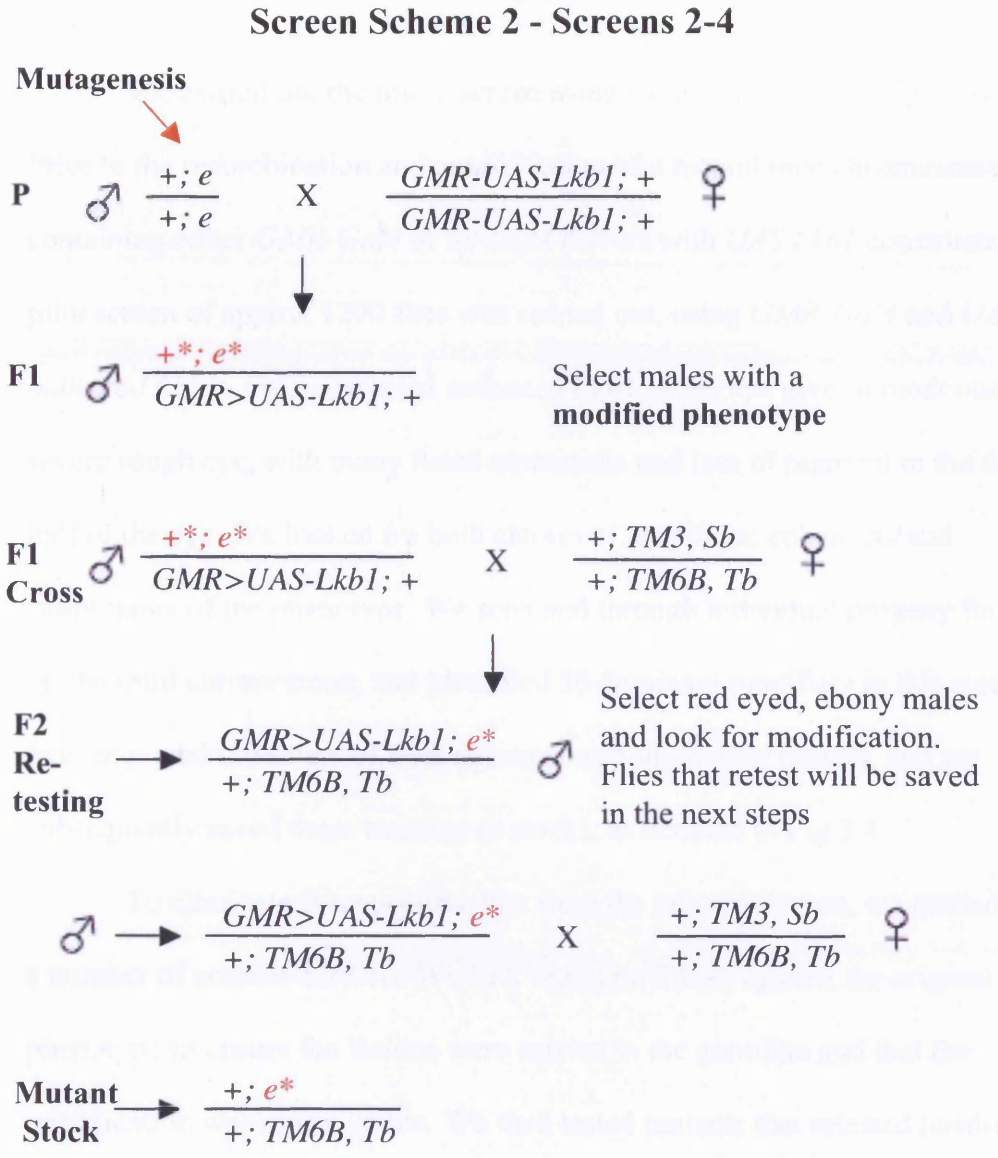



**Fig. 3-4 Schematic for primary screens (cont)** (B) Screen scheme for Screens 2-4. *Activated, kinase dead* or *RNAi Lkb1* females were crossed to mutagenised male stocks as indicated in the scheme. Male progeny were assayed for the ability to modify the tester phenotype, and individual lines were established.. At the same time, modifier lines were tested for mosaicism, by recrossing to flies with the original screen phenotype.

### 3.4.2 Pilot screen

We carried out the initial screen using Genetic scheme 1 [Fig. 3.4]. Prior to the recombination and amplification of a recombined chromosome containing either *GMR-Gal4* or *ap-Gal4* drivers with *UAS Lkb1* constructs, a pilot screen of approx 1200 flies was carried out, using *GMR-Gal4* and *UAS-activated Lkb1*. Overexpressed activated Lkb1 in the eye gives a moderate-severe rough eye, with many fused ommatidia and loss of pigment in the dorsal half of the eye. We looked for both classes of modifiers; enhancers and suppressors of the phenotype. We screened through individual progeny for hits on the third chromosome, and identified 36 dominant modifiers in this screen. We recovered modifiers of both enhancer and suppressor classes, and we subsequently saved these mutants as stocks, as detailed in Fig 3.4.

To eliminate false positive hits from the primary screen, we carried out a number of counter-screens. We first tested modifiers against the original phenotype to ensure the lesions were carried in the germline and that the modification was reproducible. We then tested mutants that retested positively and robustly to *GMR-Gal4* to exclude a non-specific interaction with the *Gal4* driver; and to *ap-Gal4-activated Lkb1* to confirm an Lkb1 specific interaction in a different system [Fig. 3.5]. Modifier lines that failed to modify the original phenotype, or that modified the GAL4 phenotype alone were discarded. Following these rigorous tests, 9 mutant stocks remained from a primary screen of 1200 flies.

### 3.4.3 Screens 2-4

We carried out a further 2 small scale screens (2000 and 1200 flies) and 1 medium size screen (5000 flies) following the above protocol. We generated *Gal4 driver/UAS-Lkb1* recombined chromosomes and amplified stocks for these screens, hence genetic scheme 2 was employed [Fig. 3.4]. Screen 2 was carried out using *GMR-Lkb1 kinase dead* in the eye, and screen 3 using *apGAL4-Lkb1 activated* in the wing. We could not maintain mutants from Trial Screen 2, as many did not survive as stocks. The F1 progeny of this screen were not healthy and were not able to produce progeny, suggesting that an excess of EMS may have been inadvertently used in this screen. Finally, screen 4 was carried out using *apGal4-Lkb1 activated.*

Following subsequent retests and counter screens, 17 mutants in total from screens 3 and 4 were recovered.

| **Screen** | **Screening for** | **Number of flies screened** | **Number taken for retest** | **Number with consistent phenotype (hit rate)** |
|---|---|---|---|---|
| Trial Screen 1 (activated Lkb1) | Modifier of rough eye | 1200 | 36 (3%) | 9 (0.7%) |
| Trial Screen 2 (kinase dead Lkb1) | Modifier of rough eye | 2000 | 38 (1.9%) | - |
| Trial Screen 3 (activated Lkb1) | Modifier of apterous phenotype | 1200 | 22 (1.8%) | 6 (0.5%) |
| Trial Screen 4 (activated Lkb1) | Modifier of apeterous phenotype | 5000 | 55 (1.1%) (18 survived balancing) | 11 (0.2%) |
| **Total** | - | **9400** | **151 (1.6%)** | **26 (0.27%)** |

**Table 3-1 Summary of screen hit rates**

## 3.5 Mutants and lethal complementation analysis

Approximately 9500 progeny of mutagenised flies were examined in 4 screens for dominant modification of the Lkb1 eye and wing misexpression phenotypes. These screens yielded 26 modifier mutants, of which 6 were enhancers and 20 were supressors of the phenotype.

The modifiers varied in strength, however most were subtle to moderate modifiers of the phenotypes. Enhancers in the eye often resulted in a further loss of pigment, fusion of ommatidia resulting in a smoother surface, and a loss of interommatidial bristles. In the wing, enhancers frequently showed a complete loss of the scutellum and fluid filled blisters in the wing that probably result from a disruption of adhesion between wing epithelia.

Suppressors from the GMR screens frequently had restored pigment in the eye, and strong suppressors showed some restoration of the ommatidial array [Fig. 3.5]. Wing screen suppressors demonstrated suppression of the severe wing 180° angles, and partial to complete restoration of the scutellum and scutellar and notum bristles.

A viability assay revealed that all of the mutants are recessive lethal, indicating disruption of genes that are required for viability.

We crossed a subset of mutants to each other for complementation analysis and mutants that survived with both mutant chromosomes were counted – the number of non-tubby (non-Tb) flies was scored as a percentage

of total progeny. Since all recovered mutants were lethal, the expected percentage of non-tubby flies is 33%. Although all recovered modifier showed recessive lethality, with such small numbers, it was unlikely that many complementation groups would be recovered, and in fact of the 9 mutants tested, I isolated 1 complementation group of 2 mutant lines. The remaining modifier lines in this group represent single hits.

| | JS1 | LS1 | SS1 | KS1 | MS1 | HS1 | AS4 | BS4 | CS4 |
|---|---|---|---|---|---|---|---|---|---|
| **JS1** | X | **1.5% 1/66 1.5% 1/67** | 44.8% 13/29 | 40% 16/40 | 42.6% 23/54 | 44.4% 16/36 | 53.5% 23/43 | 45% 18/40 | 34.8% 8/23 |
| **LS1** | X | X | 51.7% 30/58 | 79.2% 19/24 | 37.2% 16/43 | 43.7% 21/48 | 37.8% 17/45 | 48.3% 14/29 | 48% 21/44 |
| **SS1** | X | X | X | 10.9% 6/55 43.2% 32/74 | 39% 17/43 | 28% 10/26 | 44.4% 24/44 | 45% 14/31 | 26.7% 16/60 |
| **KS1** | X | X | X | X | 37% 17/45 | 23.4% 11/47 | 33.3% 12/36 | 31.4% 11/35 | 46.7% 7/15 |
| **MS1** | X | X | X | X | X | 55% 35/63 | 44.8% 26/58 | 33.3% 15/45 | 38.2% 21/55 |
| **HS1** | X | X | X | X | X | X | 30.8% 16/52 | 41.2% 26/63 | 44% 11/25 |
| **AS4** | X | X | X | X | X | X | X | 37.8% 17/45 | 37.7% 20/53 |
| **BS4** | X | X | X | X | X | X | X | X | 35% 14/45 |
| **CS4** | X | X | X | X | X | X | X | X | X |

**Table 3-2 Complementation analysis of a subset of mutants**

The crosses were scored as non-Tb progeny/total progeny; the percentage represents the number of non-Tb flies, or flies which show allelic complementation. The crosses in bold indicate the 2 alleles of a single complementation group. Some crosses were repeated to check initial results.

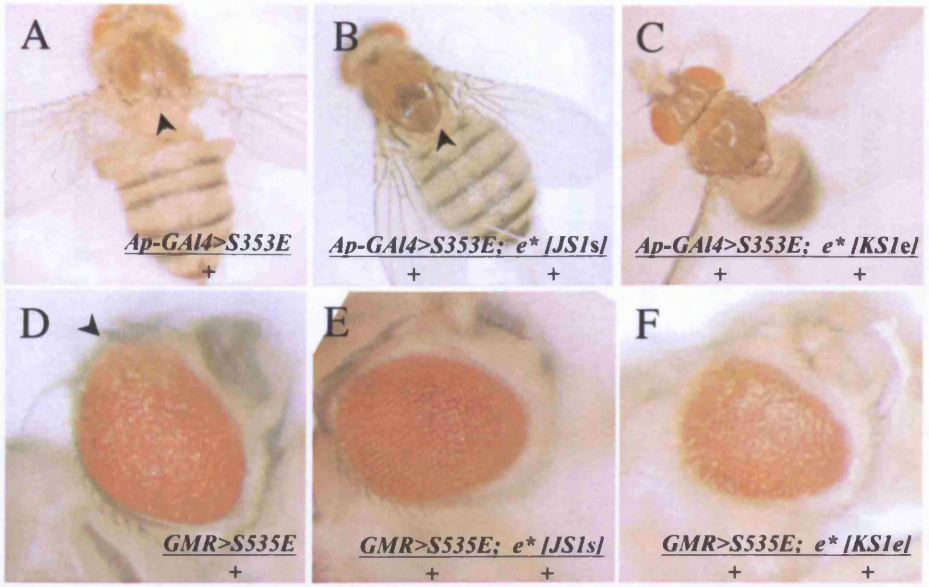



**Fig. 3-5 Screen modifiers** (A) The ApGal4>activated Lkb1 phenotype. The phenotype includes dichaete wings, where the wings are positioned almost 180° to each other; deformed scutellum (black arrowhead); and missing macrochaetae. (B) A suppressor mutant that shows restoration of the scutellum (black arrowhead), some macrochactae, and the wings are positioned at a less severe angle. (C) Enhancer mutant that shows an enhanced dichaete phenotype, whereby wings are positioned above an 180° angle, and occasionally has blisters on the wings. (D) The GMR>activated Lkb1 phenotype is a rough eye, with fused ommatidia and loss of pigment at the dorsal half of the eye. (E) A suppressor mutant shows a smoother eye with fewer fused ommatidia and no loss of pigment. (F) An enhancer mutant shows a rough eye with more fused ommatidia, and loss of pigment and bristles over a larger area of the eye.

## 3.6 A multi-tiered mapping protocol

Identifying loci and mapping molecular lesions from forward screens was traditionally carried out using deficiency kits and visible markers. Recent advances in the sequencing of fly genome have enabled comprehensive maps of molecular markers such as single nucleotide polymorphisms (SNPs) and (PCR-product length polymorphisms) (PLPs) to be generated (Berger et al., 2001; Martin et al., 2001), enabling molecular mapping using biallelic markers to a high resolution.

Both methods were utilised to begin characterising the mutants recovered from the screens described above. A brief description of these methods follows. The mapping was carried out by Ruth Wheeler and Nancy Amin.

### *3.6.1.1 Meiotic mapping with dominant markers*

Rough mapping was first undertaken using dominant markers in order to delimit the region to be SNP mapped. This is a traditional method employing meiotic recombination with marked chromosomes.

Modifier mutant males of genotype *+; $p^p$e/TM6, Tb* were crossed to females carrying a third chromosome with multiple dominant markers ($R^1$ $D^1$ $red^1$ $Sb^1$/$TM6^1$). After one round of recombination with the marked chromosome *in trans* to the multiply marked chromosome, individual males carrying the recombinant chromosome are crossed to females carrying the construct used to isolate modifiers in the screen. This cross was carried out to

screen for modification of the original phenotype and thus the presence or absence of the modifier mutation. F1 recombinant males with the modifier phenotype were then individually mated to marker females, and the F2 generation was then scored for the presence or absence of specific markers.

The absence of specific markers in these recombined chromosomes will indicate that the lesion is contained in this region. Informative recombinants that reproduce the phenotype can then be recombined once again with the marked chromosome to enable further refining of the region of interest.

Once a meiotic map position of the chromosome is identified as containing the lesion of interest, higher resolution mapping is then undertaken.

### *3.6.1.2 High resolution mapping using biallelic markers*

Using the protocol described in Berger *et al*, SNP and PLP analysis of the region of interest was carried out using fluorescently labelled primers (Berger et al., 2001). These were developed using fluorescent dye HEX, TET and FAM tags since the use of fluorescent primers will enable higher throughput.

Genome wide SNP maps have been generated by various groups (Berger et al., 2001; Martin et al., 2001; Teeter et al., 2000), however in order to characterise the mutations to a greater resolution, further SNPs were characterised around the region of interest by the sequencing of PCR products amplified from genomic DNA of the mutagenised and mapping strains. High-resolution SNP maps for these biallelic markers were generated for 3L and 3R on mutant and tester strain chromosomes, and in particular around the region of interest.

## 3.7 Discussion and conclusions

This chapter has described the generation, selection and testing of a number of mutants with putative roles in Lkb1 specific processes. Before commencing the screen, we characterised and rigorously tested a number of Lkb1 misexpression phenotypes for specificity to Lkb1 related processes, and for their ability to be modified by dominant interactions with candidate genes, such as Lkb1 itself, and Par-1. We found that misexpression of Lkb1 in the eye and wing yielded phenotypes of moderate severity that could be modified by dominant mutations in Lkb1 and Par-1.

The primary goals of this screen were to isolate and identify genes with roles in Lkb1 function and regulation. Of 9,500 mutagenised chromosomes screened, 151 individual modifier mutants were recovered. Of these, following subsequent retests to eliminate mosaic mutants and to test for reproducibility, and counterscreens to eliminate false positives that were interacting with the Gal4 system, and to test for specificity to Lkb1, 26 modifier mutants remained. Finally, I carried out complementation analysis of a subset of these mutants, and isolated one suppressor complementation group.

The mapping of these mutants will involve the use of both visible markers and molecular markers, since this will enable faster identification of mutants, as well as a more detailed analysis of critical regions in a shorter space of time.

Once critical regions are established, an examination of the region may yield a number of candidate genes, with reported functions in processes that

Lkb1 has been shown to have roles in. If mutants are available in these candidate genes, complementation analysis may be possible, or alternatively, SNP analysis to test these genes.

Although our counter-screens were designed to eliminate as many false positives as possible, unavoidable artefacts of our screen may yield false positives that were not detected using our assays: firstly, identified modifiers may interact with the products of the misexpression constructs, e.g. activated Lkb1, but not with endogenous Lkb1. Secondly, the overexpression of a kinase may lead to the activation of novel signalling pathways that are not activated by endogenous Lkb1. One way to determine this will be to recombine individual mutants onto FRT chromosomes in order to study the mutant phenotypes with respect to the Lkb1 phenotype in clones. In addition, since loss of function clones of *Lkb1* in the eye reveal defects in a number of processes, genetic interaction assays will help us determine whether the identified mutants do indeed have roles in Lkb1 specific processes.

Identified mutants can then be used in a variety of ways to understand the biology of Lkb1. Identifying specific genes and substrates of Lkb1 will enable us to further understand the processes and pathways that Lkb1 has a role in. Although functions for Lkb1 have been described in many processes, many of these have been based on overexpression studies. The identification of *in vivo* interactors will enable a greater understanding of biologically relevant and functional interactions of Lkb1. Since *Lkb1* loss of function clones in the eye have a pleiotropic phenotype, genetic interaction and epistasis experiments may also be utilised to dissect the different aspects of Lkb1 function. This will

enable us to place Lkb1 in a hierarchy of interactions, as well as identify substrates and regulators of Lkb1.

The mutants themselves will be a useful resource, since molecular characterisation of the lesions within these genes will give insight into the molecular mechanisms of Lkb1 control and regulation.

# 4 Characterisation of morphogenetic defects and cell polarity in *lkb1* mutant clones in the eye

Reported functions for Lkb1 include roles in cell polarity, cell cycle control, apoptosis, and growth (Baas et al., 2004; Karuman et al., 2001; Marignani et al., 2001; Martin and St Johnston, 2003; Ossipova et al., 2003; Qanungo et al., 2003; Spicer et al., 2003; Tiainen et al., 2002). The *Drosophila* retina is a well-suited system in which to study these processes, as these have been extensively studied in the eye (Chanut and Heberlein, 1995; Heberlein et al., 1995; Pellock et al., 2006; Penton et al., 1997). In order to study the loss of function phenotype of Lkb1 in *Drosophila*, I used the Flp/FRT system as described in section 2.1.3, and described by Golic *et al* (Golic, 1991) to create mosaic *lkb1* clones in the *Drosophila* eye.

## 4.1 The Lkb1 loss of function phenotype in the adult retina

2 alleles of *lkb1* were utilised in this study, both of which are purported nulls, and are described by Martin *et al* (Martin and St Johnston, 2003). *Lkb1 4B1-11* is a truncation mutant, with a nonsense mutation at position 98aa that produces a stop codon; *lkb1 4A4-2* is a deletion allele of 589bp, which removes 150bp of the 5' untranslated region, the start codon and the beginning of the ORF [Fig. 4.1]. Both alleles were recovered from a screen to identify mutants

that disrupt the formation of the anterior-posterior axis in the fly oocyte, and the polarity phenotypes of these alleles in the *Drosophila* oocyte were identical.

### 4.1.1 The external morphology of *lkb1* clones in the adult eye suggests defects in growth and/or apoptosis

I created mitotic clones of *lkb1* in the adult eye using the eyflp-FRT method, as described in section 2.1.3. The *eyflp* transgene is based on a fusion of the eyeless promoter with the *flpase* gene (Newsome et al., 2000). Eyeless is expressed in the eye antennal disc, and the expression patterns of this promoter have been well characterised: expression begins soon after embryogenesis, is continuous throughout larval development and terminates soon after pupariation (Quiring et al., 1994; Sheng et al., 1997). The *eyflp; 82B, w+* chromosome, when crossed to a wildtype *82B FRT* chromosome, generates clones that are marked by the absence of red pigment (Xu and Rubin, 1993). When *lkb1 4A4-2* and *4B1-11* clones were created using this method, clones were more variable in size than those created using a wildtype *FRT* chromosome, and were frequently smaller [Fig. 4.1]. This small clone phenotype was highly penetrant, and affected the majority of clones.

Smaller clones can sometimes preclude a thorough analysis of a phenotype. In order to avoid this, I utilised a *hsflp FRT* stock with a *minute* mutation on the *82BFRT* chromosome arm. *Minute* flies carry a mutation on the 82B arm that is not well characterised, although previous work has

identified lesions in ribosomal proteins, which lead to dominant effects on cell growth (Morata and Ripoll, 1975; Newsome et al., 2000). The presence of the mutation on this arm confers a growth advantage to mutant clones since the 'wildtype' clones that carry the *minute* mutation are compromised in growth, and the twin spot cells that are homozygous for *minute* die. With this stock, I generated larger clones with both alleles, enabling further analysis of the phenotype [Fig. 4.1].

The *eyflp* transgene, in combination with *minute*, further allowed me to create eyes that are over 95% mutant for *lkb1* [Fig. 4.1]. *lkb1* mutant retinas created using this method for both alleles were smaller than wildtype retinas, suggesting possible defects in growth, and/or apoptosis. Bristles were frequently disorganised, and occasionally absent. The general organisation of the ommatidia was disrupted, and ommatidia were also frequently fused to their neighbours. In addition, mutant retinas also occasionally contained small black spots in the centre of the eye, suggesting possible ectopic cell death (Kinghorn et al., 2006). Interestingly, mutant retinas created using the *4B1-11* allele displayed a more normal external morphology than those created with the *4A4-2* allele; *lkb1 4B1-11* eyes were closer to wildtype in size, and possessed fewer defects in ommatidial and bristle organisation.

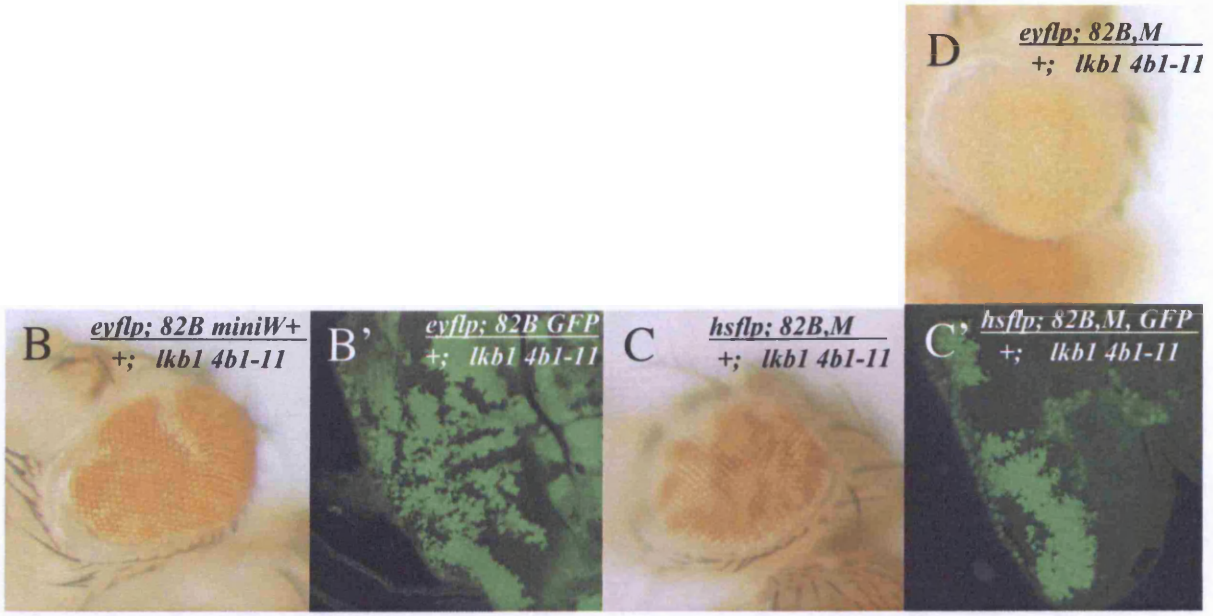



**Fig. 4-1 The *lkb1* phenotype.** (A) Schematic of 2 Lkb1 loss of function alleles. *4B1-11* is a truncation mutant; *4A4-2* is a 589bp deletion mutant, removing the 5'UTR, start codon and part of exon 1 (Adapted from Yoo *et al*, 2002). (B, B') *Eyflp* clones of *lkb1* alleles (*4A4-2* not shown) in the adult retina (B) and the larval eye disc (B') show variability in size. (C, C') *Hsflp minute* clones of *lkb1* alleles (*4A4-2* not shown) in the adult eye (C) and larval eye disc (C') also show variability in size, but are larger, encompassing most of the eye. Clones are rough, with loss of bristles and fused ommatidia. Clones also appear uneven at the surface suggesting defects in morphology or an increase in apoptosis (D) *lkb1 4B1-11 eyflp minute* adult retinas are almost entirely mutant, smaller than wild type retinas, and are rough and uneven in appearance.

### 4.1.2 *lkb1* clones in the adult retina reveal striking defects in rhabdomere morphogenesis and a frequent loss of photoreceptor cells

My initial analysis of *lkb1* clones in the adult eye thus suggested defects in growth and/or apoptosis. In addition, irregular ommatidial organisation also suggested possible defects in ommatidial development. In order to analyse these phenotypes at a single cell level, I next looked at semithin (1μm) sections of adult clones. The eyes were embedded in resin and semithin sections cut in cross-section through the eye.

The following description of the *lkb1* loss of function phenotypes was observed in clones derived from both alleles; however, *lkb1 4A4-2* clones consistently displayed a more severe phenotype than *lkb1 4B1-11* clones [Fig. 4.2].

Clones of *lkb1* in the adult eye revealed a pleiotropic phenotype, with striking defects in rhabdomere morphology (the apical domain of photoreceptors). In addition, these were frequently accompanied by a loss of photoreceptors (PRCs). Interestingly, the R7 PRC was often missing, however, other PRCs were also frequently lost from *lkb1* mutant ommatidia [Fig. 4.2].

Rhabdomeres are the apical most structure of the PRC. It is a specialised structure that is composed of approx 60,000 microvilli (Kumar and Ready, 1995), and is spherical in wildtype retinas. I found that rhabdomeres in *lkb1* clones were frequently distorted in shape, often displaying an elongated

'sausage' like appearance. In addition, the sizes of these rhabdomeres were greatly varied. Misshapen rhabdomeres have been previously observed in mutants of polarity determinants such as Crumbs, or Bazooka (Hong et al., 2003b; Pellikka et al., 2002), suggesting a possible role for Lkb1 in the regulation of cellular polarity.

The normal arrangement of the PRCs in a stereotypical trapezoidal shape was frequently compromised, and a variable number of PRCs were often missing from this arrangement. The trapezoidal arrangement of PRCs is in part dependent on the correct determination of cell fate choices in the larval disc, as specified PRCs consistently take up a predetermined position in the arrangement. Thus, this data suggests that the induction of cell fate that occurs late in larval development to specify particular PRCs may be impaired, leading to missing PRCs within the ommatidia, and subsequently, defects in PRC arrangement. Alternatively, the loss of PRCs in *lkb1* clones may be a result of ectopic apoptosis during development, and therefore defects in the organisation of the PRCs may be due to a loss of PRCs.

Other aspects of the phenotype included enlarged cell bodies, whereby the apparent basolateral portions of the cell seem greatly expanded, a phenotype, which to my knowledge has not been described before. Intriguingly, as with the loss of PRCs, I frequently observed this defect in mutant R7 PRCs [Fig. 4.2].

Finally, I occasionally observed mild defects in planar cell polarity (PCP). Ommatidia orient themselves in a stereotypical manner, and this is dependent on their position in the dorsal or ventral half of the eye. Ommatidia in the two halves of the eye have opposite chirality to each other, and in

addition, the trapezoidal ommatidia are arranged such that all the ommatidia in one half of the eye are exact images of each other. I found that *lkb1* clones exhibited multiple defects, including misrotations of the ommatidia, anterior to posterior flips, and dorsal to ventral flips [Fig. 4.2]. Although these defects were infrequently observed, it was not possible to make an accurate assessment of the penetrance of PCP defects, since the severity of other observed phenotypes preclude an accurate analysis of defects in PCP.

Since *lkb1 4A4-2* clones reveals a stronger phenotype than *lkb1 4B1-11* clones, it is possible that a small portion of Lkb1 continues to be expressed in *lkb1 4B1-11* clones that has some rescue ability. Examination of this N terminal portion did not reveal any known functional domains, however since clones derived from the *lkb1 4B1-11* exhibit less severe phenotypes, it is possible that the this segment of *lkb1* may possess a function that is important for Lkb1 function.

### *4.1.2.1 Lkb1 expressed under its genomic promoter can rescue the lkb1 phenotype in clones*

In order to test that the observed phenotypes were due to a loss of Lkb1 function, I expressed Lkb1 driven under its genomic promoter, and showed that defects in clones created from either allele, as well as lethality, can be almost completely rescued [Fig. 4.3]. This GFP-LKB1 fusion construct has previously been shown to rescue the lethality and oogenesis phenotype observed in *lkb1* mutants. Confident that the *lkb1 4A4-2* allele was a null allele, and that defects

in these clones could be attributed to the loss of Lkb1, I carried out the majority of the following analyses using this allele, unless otherwise stated.

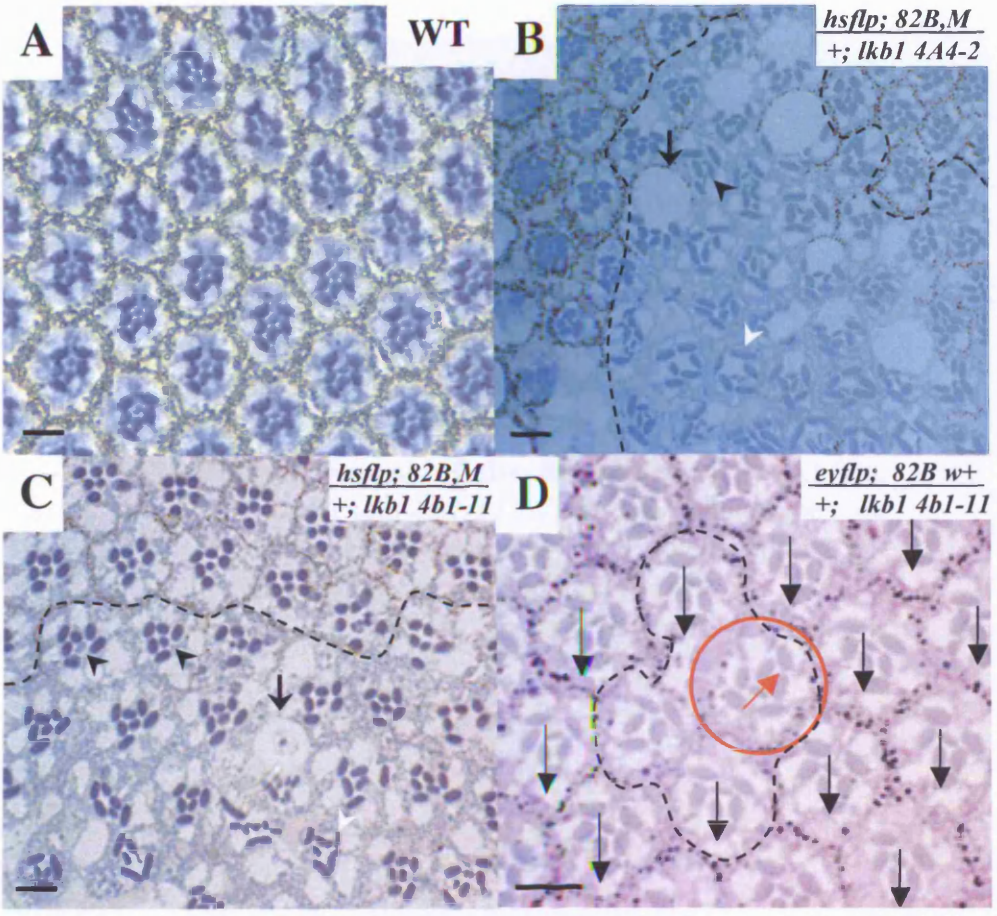



**Fig. 4-2 The Lkb1 phenotype.** (A) Light micrograph of a 1μm cross section through a wildtype retina reveals a stereotypical arrangement of photoreceptors and ommatidia. (B-D) *lkb1* clones are identified by the lack of pigment granules that normally encompass each ommatidial unit, as in (A), and are contained within dashed lines. Loss of *lkb1* leads to a loss of photoreceptors (black arrowhead), misshapen rhabdomeres (white arrowhead), enlarged cell bodies (black arrow), and planar polarity defects. *Lkb1* alleles *4A4-2* (B) and *4B1-11* (C) show the same phenotypes, but *lkb1 4B1-11* is less severe, and morphogenetic defects are less penetrant. (D) Planar polarity defect in an *eyflp 4B1-11* clone. In this image, dorsal is up, ventral is down. The circled mutant ommatidia exhibits a dorsal-ventral flip. Scale bars represent 10μm.

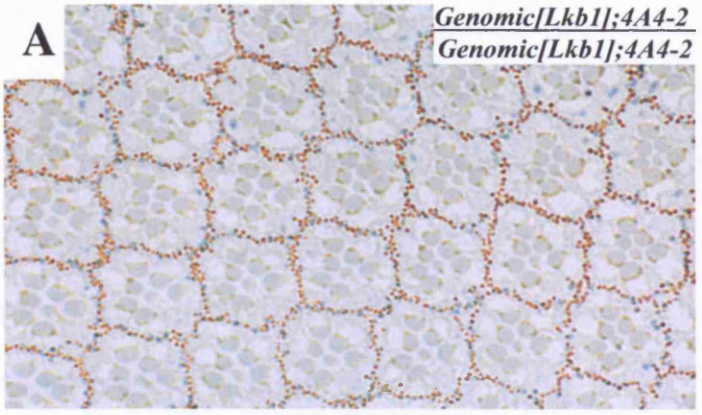



**Fig. 4-3 Rescue of the *lkb1* phenotype.** (A) The *lkb1 4A4-2* and *4B1-11* (not shown) phenotypes are almost completely rescued by the expression of full length Lkb1 under its genomic promoter.

### 4.1.3 Ultrastructure studies of *lkb1* mosaic retinas reveal further defects in photoreceptor morphology

In order to examine the morphological defects in observed in semithin sections at a higher resolution, I next looked at ultrathin (70nm) sections of adult *lkb1* mosaic retinas using a transmission electron microscope. In addition to the above defects, I also observed a number of phenotypes that have been previously detected in polarity mutants.

In addition to the defects in rhabdomere morphology previously described, I found that the sub-apical membranes of mutant PRCs were occasionally shortened [Fig. 4.4]. The sub-apical membrane, also known as the stalk domain, is the membrane region that connects the apical rhabdomere to the zonula adherens (ZA). The stalk membrane in typically characterised by infoldings of the membrane, and its development specified by polarity determinants such as Crumbs and Stardust. Previous work showed that a major feature of the *crb* phenotype in adult retinas was the shortening of the sub-apical region membrane by approximately 25% (Pellikka et al., 2002). In *lkb1* clones the sub-apical membrane occasionally lacks these infoldings of the membrane, and instead runs in a smooth line to the ZA, suggesting a similar decrease in stalk membrane length.

I also detected defects at the base membrane of *lkb1* rhabdomeres, where the presence of vesicle like structures appeared to disrupt the integrity of the base membrane [Fig. 4.4]. Similar structures were also seen in *sdt* PRCs,

and Hong *et al* demonstrated that these are overextensions of the microvilli array that constitute the rhabdomere (Hong et al., 2003b). Although *crb* mutant PRCs do not display these vesicles, Hong *et al* postulate that since Sdt is responsible for the localisation of Crb at the sub apical membrane, these defects may be a consequence of the mislocalisation of Crb in the rhabdomere, the overexpression of which has been shown to expand the apical as well as the sub apical membrane (Pellikka et al., 2002; Wodarz et al., 1995) (Hong et al., 2003b).

In wildtype TEM sections of adult retinas, junctions appear as a pair of parallel electron dense structures, appearing immediately basal to the sub apical domain. Analysis of *lkb1* PRCs revealed occasional defects in junctions. AJs appeared as ill-defined single fuzzy structures rather than the twin electron dense structures that are normally observed in wildtype retinas, suggesting that the integrity of AJs in *lkb1* clones may be compromised [Fig. 4.4].

These defects were apparent at a low frequency in *lkb1* clones, however, were consistently observed in independent clones. This suggests that *lkb1* clones may be able to recover from more severe defects earlier in development, in a similar way to *crb* mutants that can recover normal AJ structure in the adult retina despite severe fragmentation of the AJs in the pupal retina (Pellikka et al., 2002). Alternatively, *lkb1* may have a limited role in processes that affect the development of the stalk membrane, the maintenance of the basement membrane in rhabdomeres, and AJ integrity in PRCs. This data provided further indication that in addition to regulating the general morphology of the PRC, Lkb1 may have a specific role in regulating the development of different membrane domains.

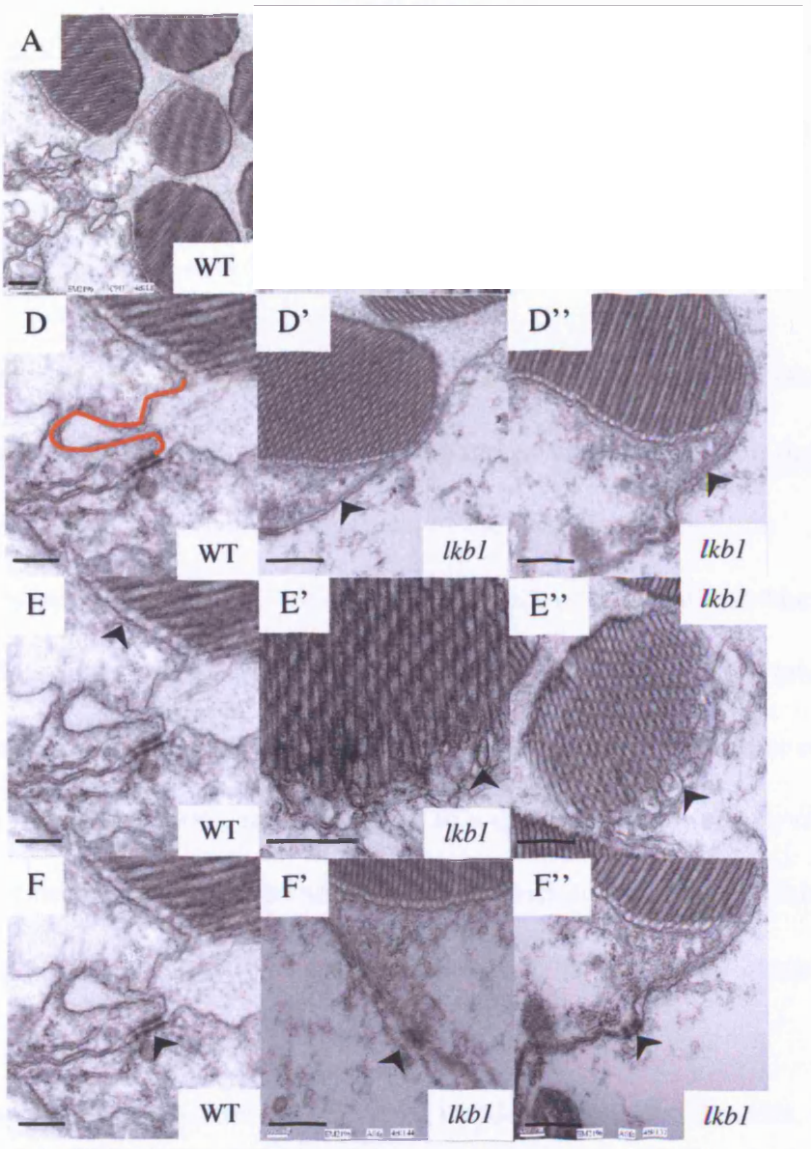



**Fig. 4.4 *lkb1* clones in the adult eye phenocopy polarity mutant phenotypes.** Ultrathin (70nm) TEM sections of *lkb1 4A4-2* clones in adult retinas. (A) Wildtype ommatidia. (B) *Sdt* mutants show an extension of the base membrane in rhabdomeres (from Hong *et al,* 2003). (C) *Crb* mutants exhibit a decrease in the stalk membrane, outlined in red (from Pellikka *et al,* 2002). (D) The wildtype stalk domain (in red) has infoldings of the membrane. (D',D'') Stalk membranes in *lkb1* rhabdomeres occasionally exhibit an apparent decrease in length and lack the membrane infoldings seen in wildtype PRCs (black arrowheads). (E) Wildtype rhabdomeres have a neat, ordered basement membrane (E',E'') *lkb1* rhabdomeres show vesicle like structures at the base membrane (black arrowheads), similar to that seen in *sdt* mutants. (F) Adherens junctions in wildtype PRCs appear as twin electron dense structures adjacent to the stalk domain. (F',F'') Junctions in *lkb1* PRCs (black arrowheads) appear as fuzzy structures, suggesting defects in AJ formation. Scale bars represent 0.5μm.

## 4.2 Loss of photoreceptor cells in *lkb1* clones in the adult eye is not due to impaired cell fate induction or apoptosis in the larval eye disc

The loss of PRCs observed in *lkb1* clones in the adult eye may be a result of either apoptosis, or defects in the cell fate induction that occurs in the third instar larval eye disc. I began my analysis of the *lkb1* loss of function phenotypes by examining *lkb1* clones in the larval eye disc. To test whether loss of *lkb1* could lead to an increase in apoptosis, a colleague in the lab stained larval eye discs containing *lkb1* clones for caspase-3, a marker of apoptosis (Xu et al., 2006). In comparison to wildtype tissue in the larval eye disc, *lkb1* clonal tissue revealed an increase in punctate caspase-3 staining, suggesting a general increase in the incidence of apoptosis in *lkb1* clones [Fig. 4.5].

Since *lkb1* clones show an increased incidence of apoptotic cells, it was possible that the loss of PRCs observed in *lkb1* clones in the adult eye were due to PRCs undergoing cell death in the larval disc. In order to test this, I stained larval eye discs containing *lkb1* clones with a number of PRC specific markers. In addition, I also wanted to exclude the possibility that missing PRCs in the adult *lkb1* retina were due to a defect in cell fate induction, and to test whether defects in PRC organisation could be due to the presumptive ommatidial cluster undergoing morphogenesis without the full complement of PRCs.

PRCs are specified in late larval development, and exhibit differential expression of neural markers, and therefore particular PRC subtypes can be highlighted by staining for these in the larval eye disc.

Spalt was used to mark the R3 and R4 PRCs (Mollereau et al., 2001); Prospero to mark the R7 PRCs (Kauffmann et al., 1996); Boss to highlight the R8 (Kramer et al., 1991); Bar to mark PRCs 1 and 6 (Higashijima et al., 1992) and finally Rough to mark R2 and R5 (Kimmel et al., 1990). I examined larval eye discs containing *lkb1* clones using these markers, and found that the full complement of PRCs are present in *lkb1* mutant clones, and correctly specified. This data demonstrates that the loss of *lkb1* in the larval eye disc does not lead to defects in cell fate, or the appropriate recruitment of PRCs in the imaginal eye disc [Fig. 4.5]. In addition, since the loss of PRCs is a highly penetrant phenotype in the adult but is not observed in the larval disc, this data indicates that the loss of PRCs in *lkb1* clones occurs during pupal development.

Bar highlights the PRC pair R1 and R6. These have a specific alignment in the eye precursor, and misorientation of this PRC pair or disruption of the regular array of PRCs is readily revealed by Bar staining. During differentiation, PRC clusters begin to rotate to achieve their final position [Fig. 1.4]. Bar staining in *lkb1* clones revealed occasional defects in PCP patterning; these PCP defects appear to occur at a greater frequency in clones in the larval eye disc than in the adult, suggesting that the full extent of PCP defects may be masked by apoptosis at a later stage. Indeed it is possible that the loss of organisation in these cells eventually leads to cell death [Fig. 4.5].

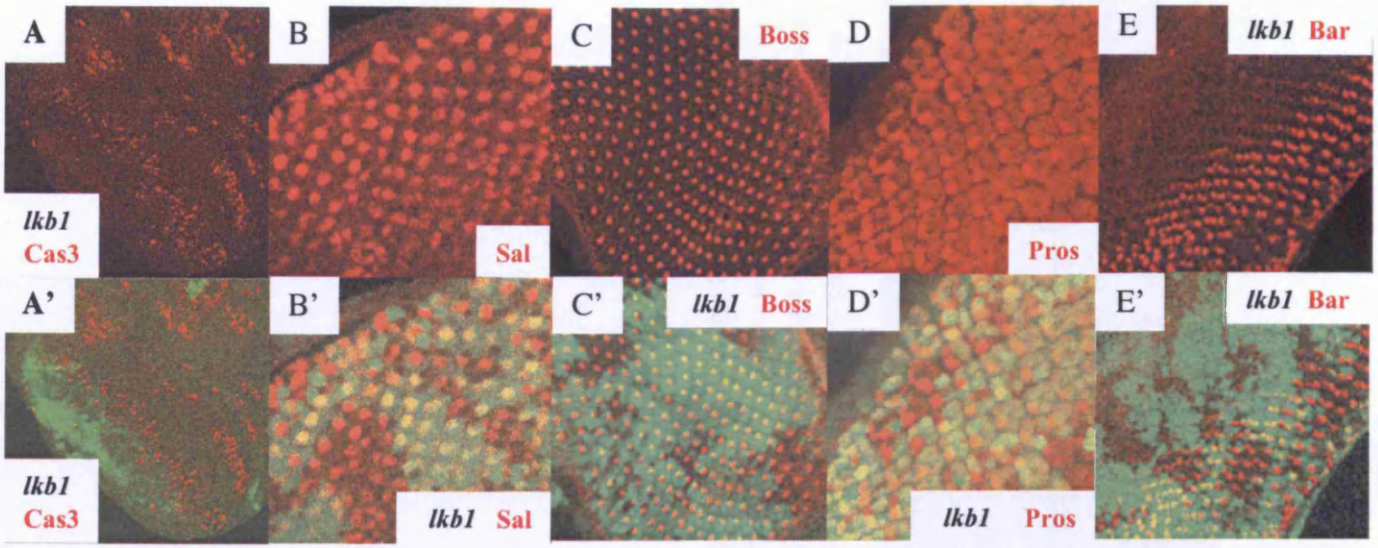



**Fig. 4-5 Lkb1 does not affect the initial commitment to a photoreceptor fate.** GFP marks wildtype tissue in all panels (A, A') Caspase-3 staining (red) of larval eye disc containing *lkb1 4A4-2* clones in the eye disc appear to show an increase in apoptosis in *lkb1* clones (experiment conducted by Nancy Amin). Neuronal markers to stain individual photoreceptor cells (PRCs) in the third instar imaginal eye disc show that PRCs 1-8 are correctly specified and positioned in *lkb1 4A4-2* clones. (B, B') Spalt (red) marks R3 and R4; these are correctly determined and specified (C, C') R7 is marked by Prospero (red); R7 PRs are correctly specified and present in *lkb1* clones. (D, D') R8 is marked by Boss (red); R8 PRs are present in clones (E, E') Bar (red) marks PR 1 and 6, and expression is normal in *lkb1* clones. Data not shown, Rough marks R2 and R5; Rough stained *lkb1* clones show that these are correctly specified.

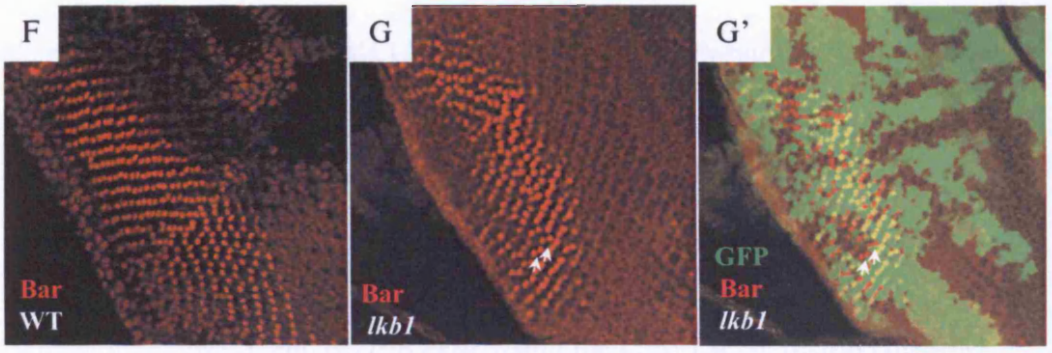



**Fig. 4-5 (cont) *lkb1* clones in the larval disc show mild planar cell polarity defects.** GFP marks the wildtype tissue in all panels. (F) Bar (red) marks PR 1 and 6, and in wildtype larval eye discs, forms an arrowlike pattern. (G,G') *lkb1* clones show occasional planar cell polarity (PCP) defects (white arrowheads).

## 4.3 Lkb1 is expressed ubiquitously at the cortices of the cell

In order to examine the localisation patterns of Lkb1 in ommatidial precursors, I stained third instar discs containing *lkb1* clones with an antibody I had raised against *Drosophila* Lkb1 (section 2.1.2.4). Previous studies examining Lkb1 localisation in different systems have reported cortical localisation, as well as localisation to the nucleus (Ghaffar et al., 2003; Karuman et al., 2001; Martin and St Johnston, 2003; Smith et al., 1999; Tiainen et al., 2002), and Martin *et al,* using the GFP-LKB1 fusion construct expressing Lkb1 under its endogenous promoter showed that *Drosophila* Lkb1 localised to the cortical domain in germline and follicle cells.

Staining in the wildtype tissue of larval discs containing *lkb1* clones, and in wildtype discs showed a weak signal of uniform localisation at the cortex of the ommatidial preclusters. *lkb1* clones showed a decrease in staining demonstrating that the signal was specific to Lkb1, however significant staining persisted within the clones, indicating either a perdurance of preclonal Lkb1 protein, or background staining [Fig. 4.6].

Since staining for endogenous expression of Lkb1 yielded a very weak signal, I decided to look at overexpressed Lkb1 in larval eye discs to better examine its localisation in the *Drosophila* retina. Using the dLkb1 antibody, I looked at the overexpression patterns of 4 different *UAS-Lkb1* constructs [Fig. 4.6]. These constructs were driven using the *GMR-Gal4* driver, therefore expression was restricted to differentiating cells, posterior to the furrow.

I found that all overexpressed Lkb1 constructs showed ubiquitous localisation at the membrane, strongly co-localising with actin at the periphery of the ommatidial preclusters. Martin *et al* showed that the cortical localisation of Lkb1 in the germline was essential for Lkb1 function, since a mutant that localised to the cortex at much lower levels was not able to rescue the *lkb1* phenotype when expressed at endogenous levels (Martin and St Johnston, 2003).

This data is in agreement with previous work demonstrating the cortical localisation of Lkb1 in diverse systems. Moreover, these observations are consistent with a role for Lkb1 in regulating epithelial polarity, since the cortical localisation of Lkb1 is essential for its function, and given that many polarity proteins have also been shown to associate with the cortex, would thus be accessible to regulation by Lkb1.

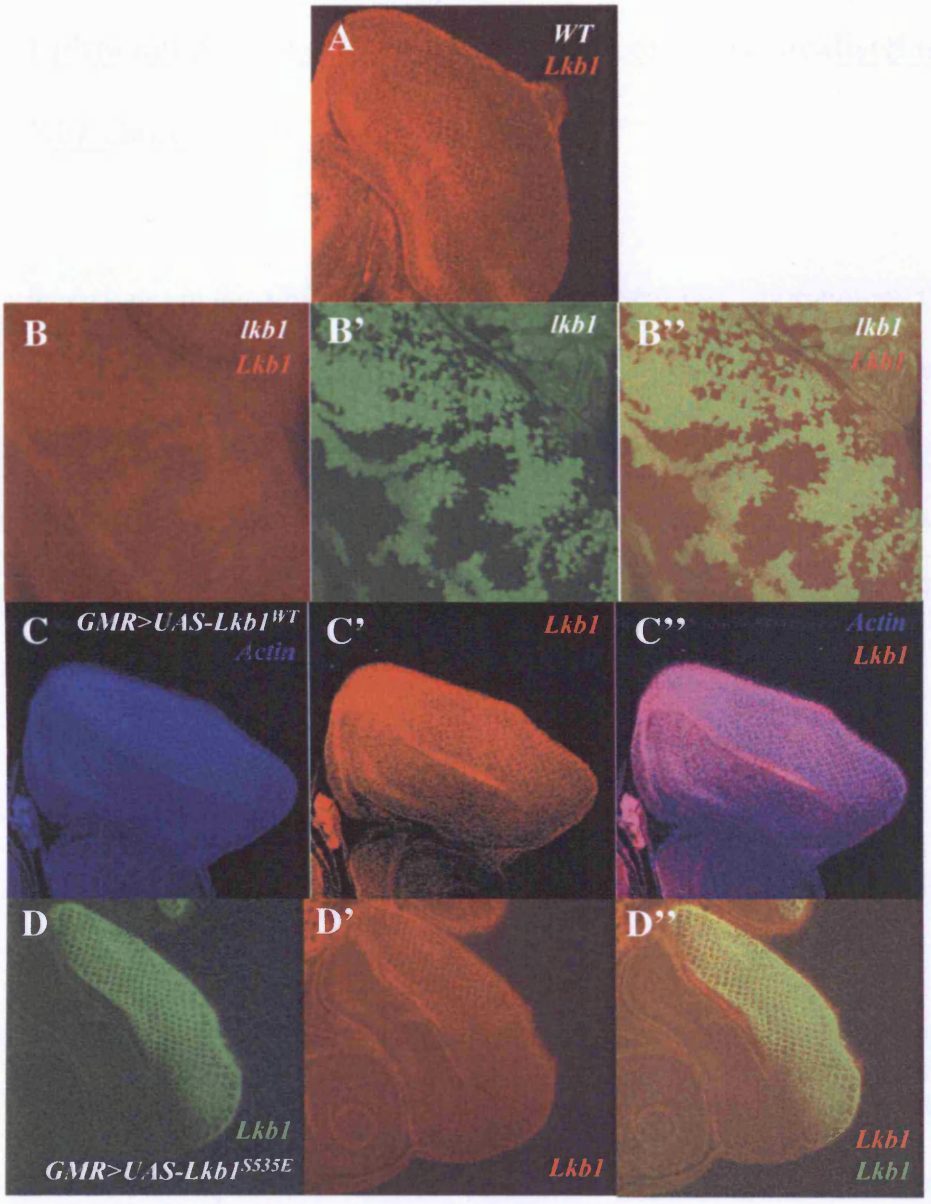



**Fig. 4-6 Lkb1 protein localises to the cortex** (A) Lkb1 antibody stain (red) in the wildtype eye disc shows a faint localisation around the developing PRC cluster and at the cortex of the PRCs. (B, B', B'') GFP (green) marks wildtype tissue, and the absence of GFP marks the presence of a clone. The expression patterns observed in wildtype tissue are specific to Lkb1, since staining decreases in *lkb1 4A4-2* clones. (C, C',C'') Lkb1 (red) localises with actin (blue) at the cortex in eye discs overexpressing wildtype Lkb1 (D'') GFP-tagged activated Lkb1 (green), when overexpressed in the eye show the same expression patterns. All other overexpression constructs (kinase dead Lkb1 (K174M), and phosphorylation dead Lkb1 (S535A) also showed uniform cortical staining (data not shown).

## 4.4 Epithelial polarity determinants are correctly localised in *lkb1* clones in the larval eye disc

*lkb1* clones in the adult retina displayed abnormal PRC morphology that was reminiscent of defects seen in polarity mutants. Having determined that Lkb1 localises to the cortex in the larval eye disc and thus may have a role in regulating cell polarity, I next looked at cell polarity in *lkb1* clones in the larval eye disc. Additionally, I wanted to test if morphogenetic defects in *lkb1* PRCs originate in the larval disc.

Morphogenetic abnormalities in rhabdomeres are suggestive of a disruption in the regulation or localisation of polarity components; mutants of polarity determinants such as members of the SAR complex Crumbs, Stardust and Patj, and members of the Par complex aPKC, Bazooka and Par-6 also show similar defects in rhabdomere formation, resulting in mutant rhabdomere shapes which are often elongated, split, bulky or fused with other rhabdomeres (Hong et al., 2003b; Nam and Choi, 2003; Pellikka et al., 2002; Richard et al., 2006).

I examined the localisation of Baz and DaPKC, components of the apical Par complex, and Armadillo (Arm), a component of the adherens junctions (AJ) and the *Drosophila* orthologue to β-catenin, in *lkb1* clones in the larval epithelium to see if epithelial polarity was disrupted. In wildtype eye discs, DaPKC localises to the apical membrane, while Arm and Baz are observed immediately basal to DaPKC.

Lateral confocal sections of larval eye discs containing *lkb1* clones showed that all three components were localised to the correct domains, and that the columnar morphology of *lkb1* clones in the disc appeared normal [Fig. 4.7]. In addition, cross section profiling of Arm and actin staining in larval eye discs containing *lkb1* clones also revealed a normal distribution in the localisation of these components, demonstrating that epithelial polarity is intact in the absence of *lkb1* in the larval eye disc [Fig. 4.7]. These results suggest that defects in PRC morphology occur at a later stage in development, since key components of the polarity network are correctly localised, and the morphology of the larval epithelium is correctly maintained in *lkb1* mutant clones.

Thus, using markers in the third instar larval disc for specific PRCs, and markers for epithelial polarity, I determined that the morphological defects and loss of PRCs observed in adult *lkb1* clones do not originate in the larval eye disc. Since the larval eye disc subsequently undergoes dramatic changes in morphology in early pupal development, it is possible that there is a dynamic requirement for Lkb1 in these processes, and defects do not become apparent until this stage.

Alternatively, the absence of polarity defects in the *lkb1* larval disc may be a consequence of perdurance of preclonal Lkb1 protein, since Martin *et al* (Martin and St Johnston, 2003) also demonstrated that Lkb1 protein could persist for several days after the induction of *lkb1* germline clones. However, loss of *lkb1* in the larval eye disc resulted in an increase in apoptosis as well as mild PCP defects, arguing against perdurance of Lkb1 in the larval eye disc.

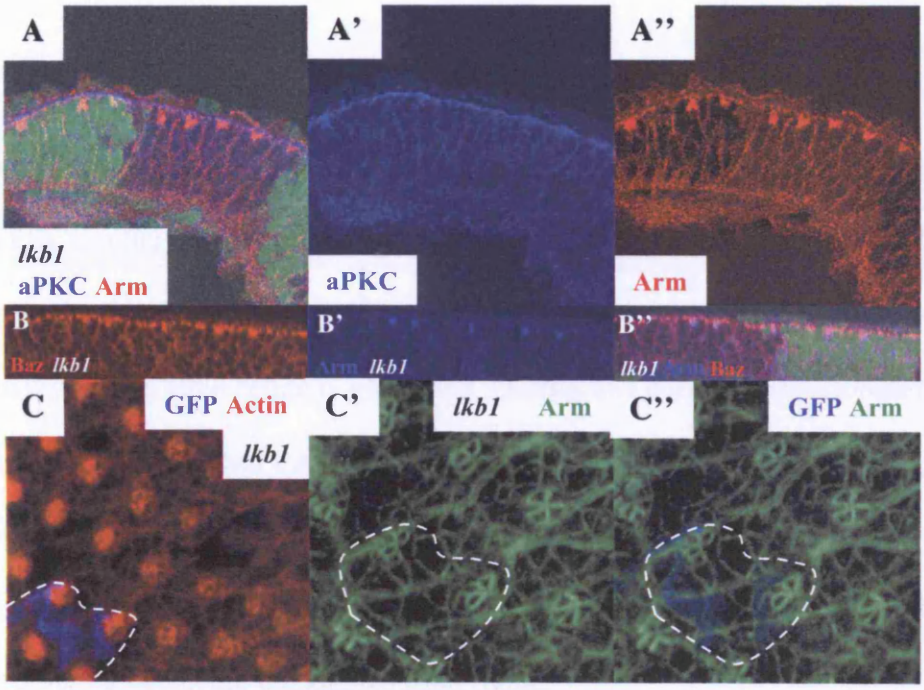



**Fig. 4-7 Characterisation of cell polarity in *lkb1* clones in the third instar eye disc.** GFP marks wildtype tissue (A-B'') Polarised components are correctly localised in *lkb1* clones. (A,A',A'') Mosaic discs stained with Arm (red) and aPKC (blue) show correct localisation of apical polarity determinants. (B,B',B'') Baz (red) also shows correct localisation in *lkb1* clones. (C,C',C'') GFP (blue) and the area contained within the dashed white lines mark the wildtype tissue. Actin (red), and Arm (green) show normal expression patterns in *lkb1* clones.

## 4.5 Lkb1 is required for the morphogenesis of photoreceptor cells during pupal development

Having established that there are no detectable defects in epithelial polarity in *lkb1* clones in the larval eye disc I next examined the morphology of the PRCs in the pupal retina. Morphogenesis of the larval eye disc takes place during the pupal developmental stages, in the course of which the crystalline structure of the adult retina is generated. During this period of development, the apical membrane of the PRC undergoes extensive growth to produce the adult rhabdomere. Since the pupal retina undergoes such dramatic morphogenetic changes, I reasoned that defects in PRC morphology might therefore be occurring during this time frame.

The first evidence of the rhabdomere structure in the pupal retina are distinct puncta that become apparent from approximately 10% pupal development (pd). This structure stains intensely for actin, highlighted by phalloidin, which stains the rhabdomere structures, as well as the cell cortices. This structure persists through to between 30 and 40% pd, at which stage, the adult rhabdomere structures begin to form (section 1.4.2.2), and distinct rhabdomeres for each PRCs begin to become apparent. PRCs in each ommatidia begin to be assembled into the stereotypical trapezoidal arrangement seen in adult retinas. In cross section images, actin rich structures can be seen to expand as microvilli stacks are assembled, to form the spherical rhabdomere seen in adult PRCs.

I began my analysis of rhabdomere structure and PRC morphology by looking at pupal clones of both *lkb1* alleles. In keeping with previous data, I found that the *lkb1 4A4-2* allele exhibited more severe defects in rhabdomere morphogenesis. Since the *lkb1 4A4-2* allele is a deletion mutant that results in complete loss of Lkb1, and the defects can be completely rescued by Lkb1 expressed under its genomic promoter, I primarily used this allele to study the morphological defects more closely.

I looked at a number of different stages of pupal development, from 10 - 55% pd. In *lkb1* clones in 10 and 20% pd retinas, I observed defects in actin localisation at the apex of the ommatidia [Fig. 4.8], indicating a possible role for Lkb1 in organising the actin cytoskeleton in pupal retinas. Actin is normally localised in a continuous band along the apical side of the retina, however, in early *lkb1* pupal clones, actin staining was present at the apical membrane at irregular intervals and was also often significantly decreased in intensity.

Additionally, at 10 and 20% pd, mutant ommatidia clusters were often no longer concentrated at the distal region of the retina, and frequently had the appearance of 'falling' into the developing retinal floor. Cross section confocal images of 20% pd *lkb1* clones showed that developing ommatidia that were mutant for *lkb1* lacked the regular size and shape seen in wildtype ommatidia. The actin rich spot in the centre of the ommatidia was frequently missing, and this is possibly due to the ommatidia that are observed to be falling out of the retinal plane in longitudinal confocal images. In addition, I also observed severe defects in cone cell morphology, including a loss of cone cells, and fusion between separate cone cell clusters. These defects in cone cell

configuration and in the attachment of the mutant ommatidia to the cone cell roof is discussed more fully in section 5.6.

At 30% pd, these defects were still apparent; in some instances there was an apparent fusion of ommatidia, the size and shape of the ommatidia varied from the uniform size of wildtype ommatidia, and were no longer packed in a regular array. At 40% pd, the loss of PRCs began to be apparent since individual PRCs can be distinguished. At this stage, 7 PRC bodies are normally apparent, however 40% pd *lkb1* ommatidia had a variable number of PRCs missing from the cluster. In addition, mutant PRCs also had abnormal cell shapes [Fig. 4.8].

By 55% pd, rhabdomeres showed gross defects that include distortion of shape e.g. elongated rhabdomeres, and the fusion of rhabdomeres from multiple ommatidia [Fig. 4.8].

These results show that Lkb1 is necessary for the development of rhabdomeres, for maintaining the integrity of distinct ommatidia as separate units, and for the proper localisation of the ommatidia at the distal region of the retinal epithelium in young (10-20% pd) retinas. Since many of these processes are dependent on the correct polarisation of cells in the epithelium, this data suggests a role for Lkb1 in maintaining epithelial polarity. Indeed, Martin *et al* demonstrated that loss of *lkb1* in follicular epithelial cells could lead to defects in the localisation of aPKC and Arm, 2 key polarity determinants in epithelial cells (Martin and St Johnston, 2003).

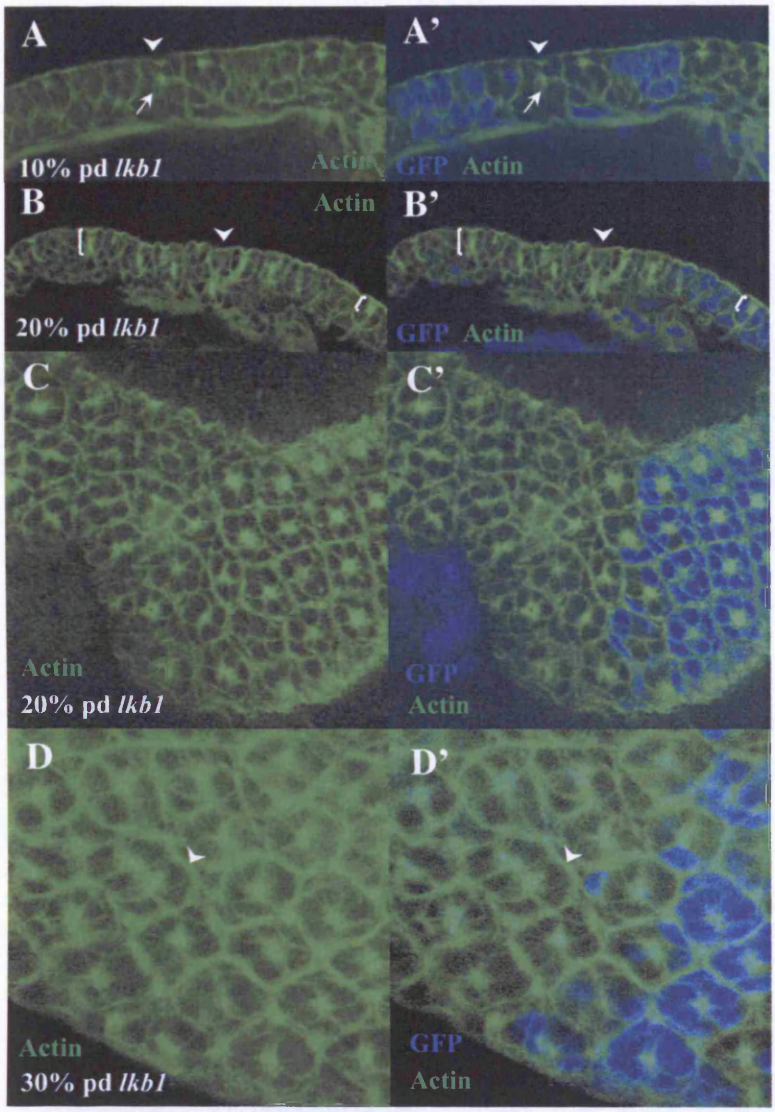



**Fig. 4-8 Defects in actin and rhabdomere morphology in *lkb1* clones are apparent in the pupal retina** GFP (blue) marks wildtype tissue, and actin is in green. (A,A') At 10% pupal development (pd), ommatidial clusters in *lkb1* clones lose their apical position in the epithelium (white arrow), and actin localisation is disturbed and decreased in expression at the apical surface of the epithelium (B-C') 20% pd *lkb1* clones show defects in ommatidial positioning as well as defects in morphology such as ommatidial length and shape (scale bars). Actin is decreased at the apical surface of the epithelium (white arrow head). (C,C') Cross section views of 20% pd clones show structural defects in *lkb1* ommatidia (D, D') Clones in 30% pd retinas show similar defects, including fused ommatidia, and abnormal ommatidium shapes (white arrowhead) .

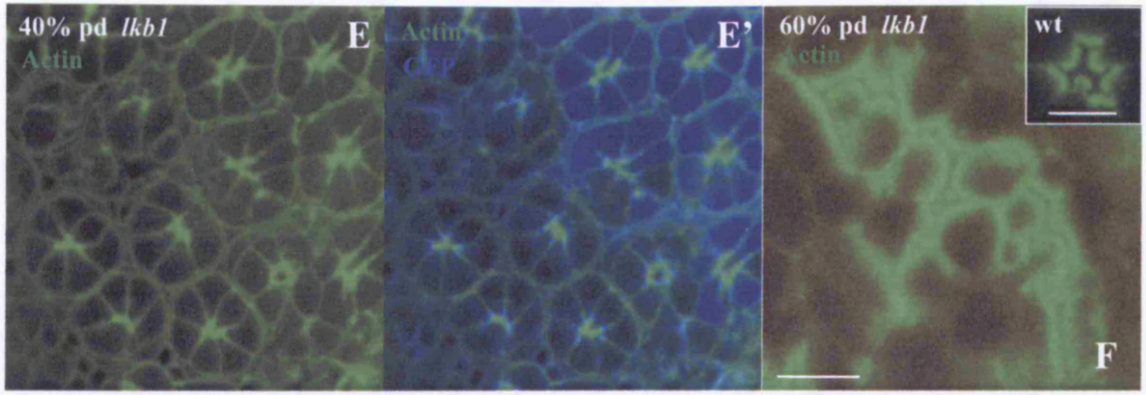



**Fig. 4-8 (cont) Defects in actin and rhabdomere morphology in *lkb1* clones are apparent in the pupal retina.** GFP (blue) marks wildtype tissue. Actin is green. (E, E') 40% pd retina. Actin rich structures resembling the adult rhabdomere first begin to become apparent at approx 40% p.d. It is at this stage that structural defects in individual rhabdomeres and the loss of rhabdomeres first become apparent. (F) By 60% pd the defects are striking, and include fused ommatidia, and elongated and misshapen rhabdomeres. These defects are more severe and more penetrant in *lkb1 4A4-2* clones. Scale bars represent 5μm.

## 4.6 The SAR complex determinants are mislocalised from the subapical membrane in *lkb1* clones in the pupal retina

Since the correct development of the PRCs has been shown to be dependent on intact cellular polarity in the PRCs (Hong et al., 2003b; Izaddoost et al., 2002; Nam and Choi, 2003; Pellikka et al., 2002; Richard et al., 2006), I next decided to look at the localisation of polarity determinants and members of the SAR complex: Crumbs (Crb), Stardust (Sdt) and DPATJ. The dynamic localisation of polarity effectors is well documented in the fly eye, particularly during the pupal stages, and the subcellular distribution of these components in the PRCs provide a readout of the polarity status of the cell (section 1.5.2). In addition to the defects in rhabdomere formation, the parallels in the adult *lkb1* eye phenotype to the *crb* phenotype prompted me to begin by examining the localisation of the transmembrane polarity determinant Crb during pupal development. I used Arm as an additional marker to mark the ZAs and membranes.

### 4.6.1 Crumbs

Crb exhibits a dynamic localisation throughout the development of the eye; in the larval disc, Crb localises to the marginal zone, a site immediately basal to the apical domain, and just adjacent to the ZA. During pupal development, Crb localises to the developing stalk domain, which is the

membrane that connects the apical and junctional domains, and this specific localisation is maintained through to adult PRCs.

I found that Crb localisation was frequently disrupted in *lkb1* clones in the pupal retina. This disruption begins between 30% pd and 40% pd, since the apical localisation of Crb in 30% pd *lkb1* clones is unperturbed [Fig. 4.9]. At 40% pd, the period during which polarised markers for the apical and sub apical domains become resolved, Crb shows an apparent basal spread and can often be seen on the basolateral membrane. In wildtype ommatidia, the Crb signal can be resolved from Arm by 40% pd, however, in *lkb1* ommatidia, Crb sometimes co-localises with Arm, suggesting that the maintenance of distinct membrane domains is impaired in *lkb1* clones [Fig. 4.9]. These defects persist into the late pupal stages; however, it was revealing that the abnormal distribution of Crb did not coincide with every incidence of aberrant morphology, since this suggests that the mislocalisation of Crb is a secondary effect of the morphological defects, and not a causal one.

Additionally, other aspects of the *crb* phenotype were not reproduced in *lkb1* retinas, providing further evidence that despite phenotypic similarities, the morphogenetic defects in *lkb1* clones are not primarily due to defects in Crb localisation. Mutant PRCs in *crb* mosaic eyes are fragmented and disjointed along the proximo-distal axis, and are bulkier towards the 'top' or distal part of the eye. In addition, rhabdomeres extend only 40-60% of the normal length along the proximo-distal axis (Pellikka et al., 2002). Longitudinal immuno-fluorescence images of pupal retinas and adult light microscopy sections showed that *lkb1* PRCs have no defects in this proximo-distal extension, although they do exhibit fragmentation defects along the length of the PRCs.

Unlike *crb* mutants, *lkb1* mutant rhabdomeres are able to fully extend along the proximo-distal axis, and are uniform in density along their lengths [Fig. 4.9].

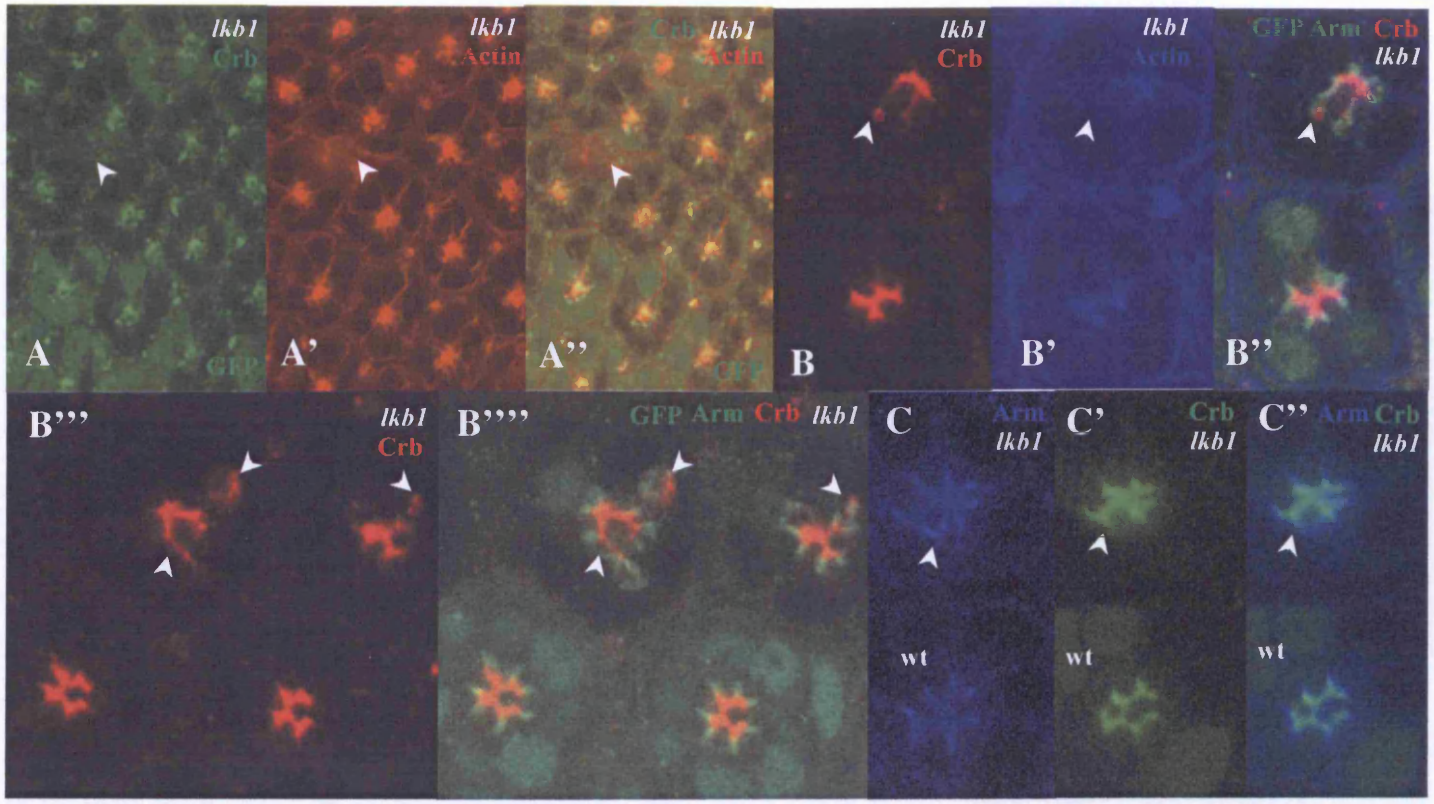



**Fig. 4-9 Crumbs is mislocalised in *lkb1* clones.** GFP marks wildtype tissue (A-A'') 30% pd retinas containing *lkb1 4A4-2* clones do not show defects in Crb localisation, despite defects in actin (red) (white arrowhead) (B-B''') At 40% pd *lkb1* ommatidia show defects in Crb localisation. Crb is normally localised apically to Arm (green), a junctional marker. Basal expansion of Crb expression is frequently seen, and Crb is sometimes seen basal to Arm (green) (white arrowheads). In addition, overlap of Crb and Arm are also frequently observed. (C) Crb localisation often remains correctly positioned in PRCs with aberrant morphology and despite defects in Arm localisation (white arrowhead).

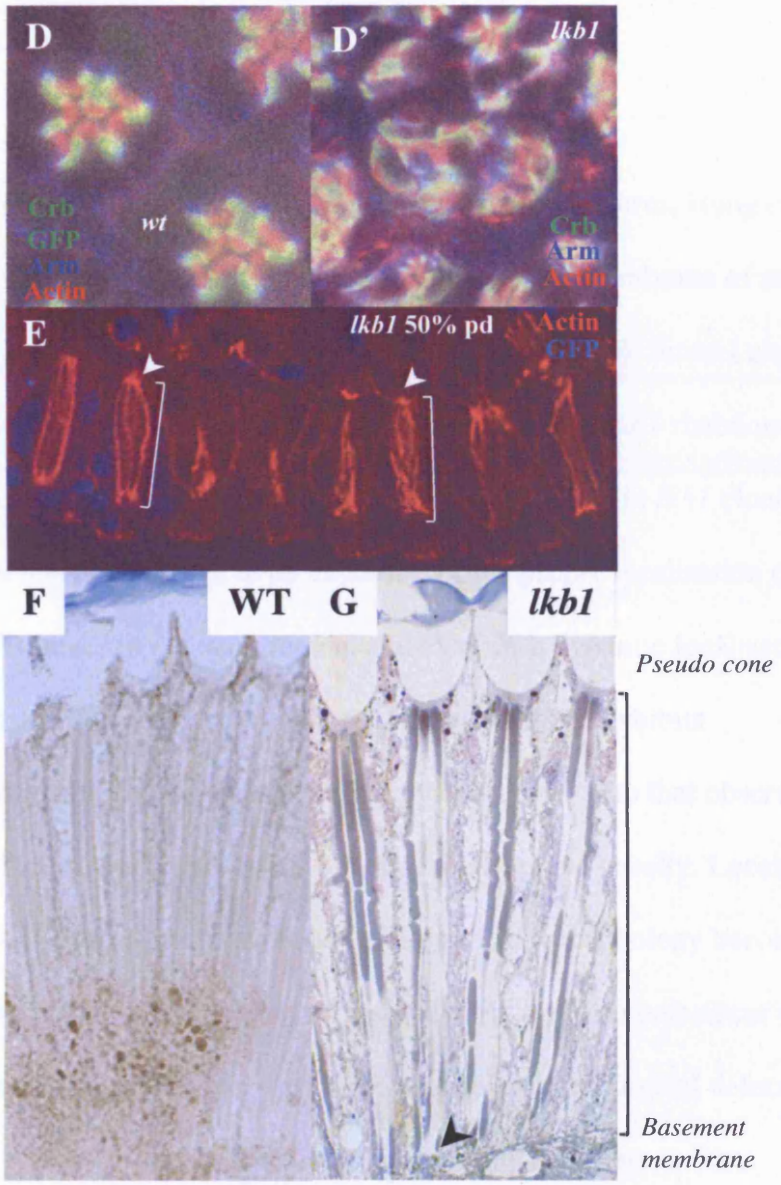



**Fig. 4-9 (cont) Crumbs is mislocalised in *lkb1* clones.** (D, D') Mosaic ommatidia at 55% pd, *lkb1* clones at 55% pd show severe defects in Crb localisation (green) (E) *lkb1* ommatidia show defects in morphology, but undergo normal proximo-distal extension (white scale bar), and rhabdomere feet remain attached to the basement membrane (white arrowhead) (F, G) Adult *lkb1* rhabdomeres show breaks throughout the proximal-distal length of the rhabdomere, but extend normally to the basement membrane (black arrowhead).

### 4.6.2 Stardust

In addition to morphological defects in *sdt* rhabdomeres, Hong *et al* also noted an overextension of the microvilli at the base membrane of mutant rhabdomeres, which resembled vesicles (Hong et al., 2003b). Since I also occasionally observed a similar defect in TEM analysis of *lkb1* rhabdomeres, it was possible that defects in Sdt localisation were occurring in *lkb1* clones. In addition, Sdt has been shown to be essential for the proper localisation of Crb.

Sdt localises in the stalk region, and exhibits a dynamic localisation, similar to that shown by Crb. At 40% pd, I found that Sdt exhibits mislocalisation defects in clones deficient for *lkb1*, similar to that observed for Crb, where the normally sub-apical Sdt appears to spread basally. Localisation defects in Sdt become more severe as defects in PRC morphology become more severe in older clones. However, as with Crb, these defects occur with varying penetrance, and do not always accompany morphological defects in the rhabdomeres, indicating that these defects are likely to be due to the morphological changes or other primary defects in polarity, rather than a cause of it [Fig. 4.10].

### 4.6.3 DPATJ

Finally, of the SAR complex members, I examined the distribution of DPATJ in 40-50% pd. retinas. Since DPATJ is dependent for its localisation on

Crb and Sdt, I expected to see similar defects in DPATJ localisation. Again, I observed basal spreading of DPATJ from the sub-apical region that indicated a secondary effect, since mislocalisation was not always seen to coincide with defects in cell shape and morphology [Fig. 4.10]. In addition, I also occasionally observed decreased expression of DPATJ at the sub apical membrane in mutant PRCs.

Defects in the localisation of the SAR complex members can lead to malformed ommatidia with abnormal rhabdomeres and missing ommatidia, however immuno-histochemical analysis of these determinants in *lkb1* clones suggest that these defects are unlikely to be responsible for the loss of rhabdomere structure. Instead these defects are likely to be a consequence of morphological changes, or earlier defects in polarity precipitated by other factors.

A further intriguing piece of data to arise from these studies was the defect in Arm localisation. Arm normally localises to the junction, just basal to the stalk membrane, however, in *lkb1* PRCs, Arm frequently shows an expansion of staining, occasionally overlapping with stalk membrane markers, but in large part spreading towards the basolateral membrane. This data is discussed more fully in the next chapter.

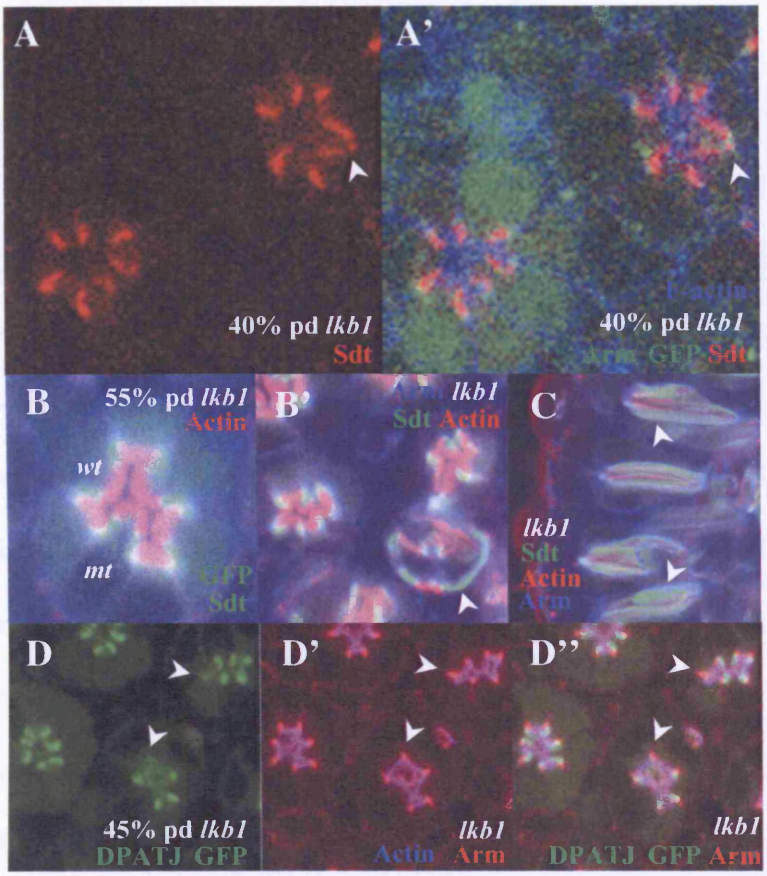



**Fig. 4-10 Sdt and DPATJ are mislocalised in *lkb1* clones**
GFP marks wildtype tissue (A, A') Sdt (red) in 40% pd retinas shows mislocalisation in *lkb1 4A4-2* clones (white arrowheads) (B) 55% pd mosaic ommatidium (B') 55% pd *lkb1* clones show severe defects in Sdt localisation (white arrowhead) (C) Side view images of 55% pd *lkb1* clones show defects in Sdt localisation - a thickening of Sdt expression is evident in longitudinal images (white arrowheads). (D, D', D'') DPATJ (green), in addition to mislocalisation, also occasionally shows decreased staining in 45% pd *lkb1* PRCs (white arrowheads).

## 4.7 Defects in cell polarity in *lkb1* clones are not due to overproliferation or ectopic cell death

Martin *et al* showed that the polarity phenotype observed in *lkb1* follicles cells was not due to cell death or overproliferation since TUNEL assays and PH3 staining failed to detect more apoptotic or mitotic cells in mutant follicle cells compared to their wildtype neighbours. To test this in *lkb1* clones in the eye, I labelled third instar larval discs with BrdU to test if there was an increase in cell division in *lkb1* clones. There was no obvious increase in BrdU labeled cells in *lkb1* clones, suggesting that there was no overproliferation [Fig. 4.11]. In support of this data, the adult semithin sections of *lkb1* clones did not reveal an excess of PRCs or other cells.

Given that an increase in apoptosis in mutant clones in both the larval disc and the pupal retina is apparent, I next wondered if excess cell death could lead to the polarity phenotype in *lkb1* clones. A colleague in the lab examined the effects of blocking cell death by expressing the pan caspase inhibitor, p35 in 45% pd MARCM *lkb1* clones. Staining with Arm showed that *lkb1* clones continue to exhibit mislocalisation, suggesting that defects in polarity are not due to excess cell death in *lkb1* clones [Fig. 4.11].

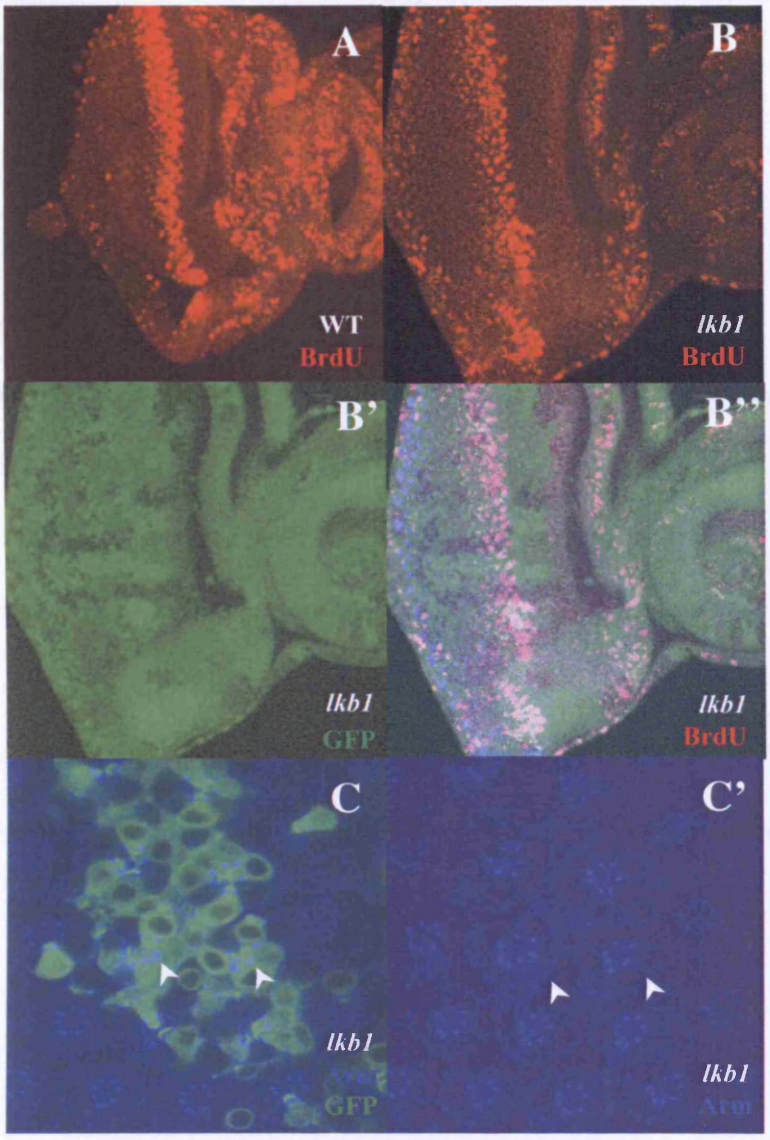



**Fig. 4-11 p35 rescues cell death but not morphogenetic defects in *lkb1* clones.** (A) BrdU stain (red) of a wildtype disc (B', B'', B''') BrdU staining of *lkb1 4A4-2* mosaic eye discs show no change in cell proliferation. (C, C') The MARCM system was used to mark *lkb1* clones (marked by GFP). Expression of p35 in *lkb1* clones showed that despite a rescue of cell death, morphogenetic defects, and defects in Arm localisation persisted (white arrowheads), indicating that apoptotic processes are not leading to the morphogenetic defects seen in *lkb1* clones (experiment by Nancy Amin).

## 4.8 Lkb1 is required for the proper localisation of Baz and DaPKC at the apical and ZA membranes in photoreceptors, respectively

Having determined that the SAR complex determinants show defects in localisation in *lkb1* clones, I next examined the subcellular localisation of the Par complex members in *lkb1* PRCs. The Par complex is a second polarity complex that localises to the apical and sub apical membranes, and is comprised of 3 members: Bazooka (Baz), DaPKC, and Par-6. Apical localisation of Baz and DaPKC localisation in the larval eye disc has been previously shown by Nam *et al* and Kempkens *et al* (Nam and Choi, 2003) (Kempkens et al., 2006), and I had previously shown that Baz and DaPKC proteins are properly localised at the apical membrane in *lkb1* clones in the eye disc. This demonstrated that polarity was correctly determined in the larval disc, prior to the morphogenesis of the pupal eye.

### 4.8.1 DaPKC

DaPKC is an apically localised determinant of polarity, and has a role in the development of different membrane domains throughout the cell. It functions through a mutually antagonistic relationship with Par-1 to determine specific membrane domains (Hurov et al., 2004), and similar, conserved interactions have been described with Lgl, and Crb (Betschinger et al., 2003;

Chalmers et al., 2005; Sotillos et al., 2004; Yamanaka et al., 2003). I examined DaPKC distribution in 40% pd *lkb1* clones, and found that in addition to an expansion in DaPKC expression in the lateral and basal parts of the cell, similar to that seen in the SAR complex proteins, DaPKC also frequently appeared to have reduced accumulation or more diffuse staining at the apical membrane. Defects in DaPKC localisation were more severe and penetrant than that seen with Crb, Sdt and DPATJ, suggesting that the regulation of DaPKC localisation by Lkb1 may be more direct. In addition, all observed instances of morphological defects in these clones were accompanied by defects in DaPKC localisation [Fig. 4.12].

#### *4.8.1.1 Loss of DaPKC enhances the morphogenetic defects in lkb1 clones*

This data, and in particular data suggesting that morphological defects in the rhabdomeres are consistently accompanied by defects in DaPKC indicate that DaPKC may be a downstream effector of Lkb1. In order to further explore this, I carried out a genetic interaction experiment to see if reducing the gene dosage of *DaPKC* by 50% could modify the *lkb1* phenotype in the adult eye. I generated clones of *lkb1* in the eye, in a genetic background that is heterozygous for the *DaPKC* null allele, *psu69*. In this genetic background *lkb1* clones, haploinsufficient for *DaPKC,* display a greater penetrance of the morphological defects, with rhabdomeres frequently displaying more exaggerated morphological defects, and a greater penetrance of other defects observed in *lkb1* clones such as loss of PRCs. From this data I suggest that

Lkb1 and DaPKC may be acting in the same pathway, and that this pathway may have a role in regulating or maintaining cellular polarity in the retinal epithelium [Fig. 4.12].

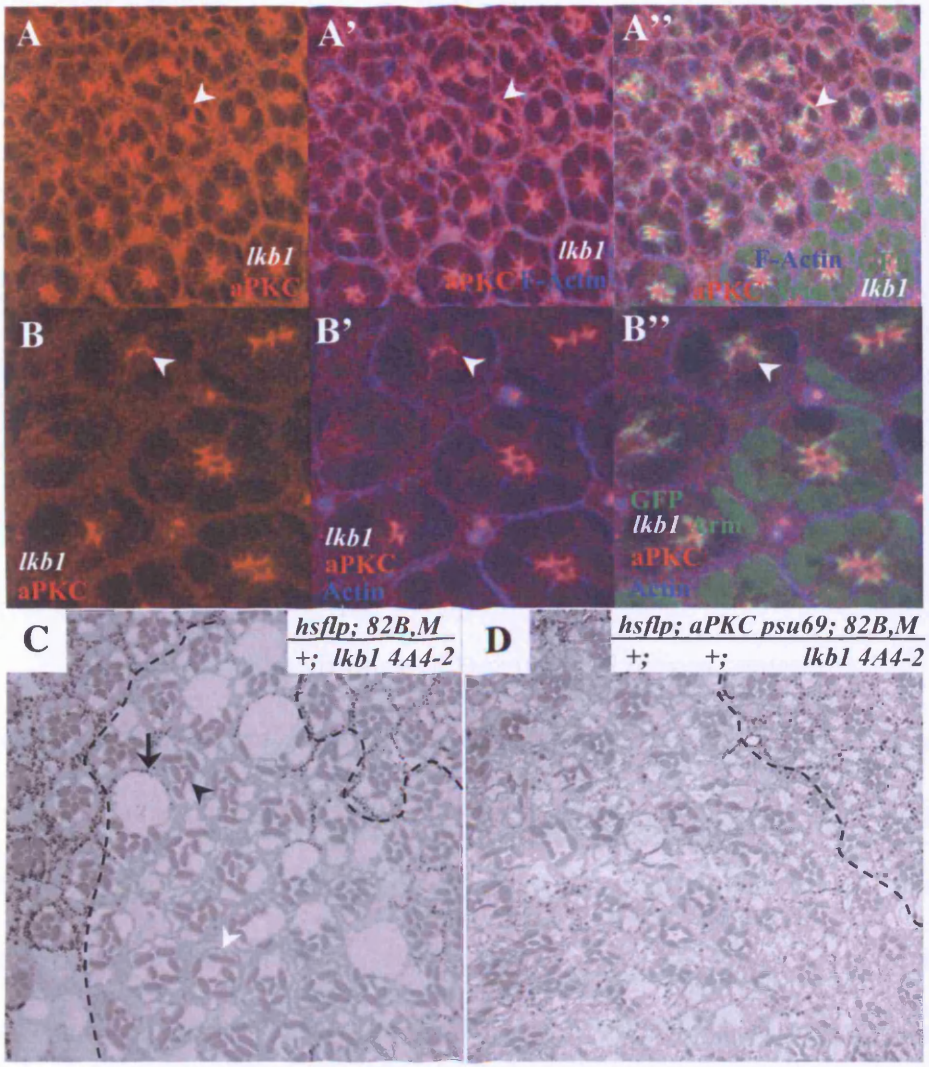



**Fig. 4-12 DaPKC shows localisation defects in *lkb1* clones, and genetically interacts with Lkb1.** *lkb1 4A4-2* clones are marked by the absence of GFP. (A-B'') DaPKC (red) is sometimes mislocalised (A) and sometimes appears to be reduced in concentration at the apical membrane in *lkb1* clones (B) (white arrowheads). (C) *lkb1* clones in the adult eye are indicated by the absence of pigment granules, and are within the dashed lines (C), or to the left of the dashed lines (D). The *lkb1* phenotype in the adult eye includes loss of PRCs (black arrowhead), misshapen rhabdomeres (white arrowhead), and enlarged cell bodies (black arrow). (D) *lkb1* clones in a genetic background heteroallelic for DaPKC exhibit more severe defects than *lkb1* clones in a wildtype genetic background. Both morphogenetic defects and cell death are increased in severity.

### 4.8.2 Bazooka

I next looked at the Par complex protein, Baz. Baz is one of the earliest effectors of polarity, and its additional role in establishing the AJs has been extensively described in the *Drosophila* embryo (Muller and Wieschaus, 1996). Unlike DaPKC and Par-6, that are briefly localised at the AJs, Baz is mainly found at the AJ, co-localising with Arm throughout pupal development. *baz* clones in the adult eye lead to defects in rhabdomere formation (Hong et al., 2003b), similar to that observed in *lkb1* clones.

I examined the localisation of Baz in pupal retinas containing *lkb1* clones, at 40% pd, a stage at which defects were seen in DaPKC staining. At this stage Baz is normally seen as 7 discrete spots flanking each rhabdomere of a single ommatidium, co-localising with the junctional marker Arm. I looked at Baz expression patterns in *lkb1* clones, using Arm as a junctional marker and additionally to determine the effects of Arm and Baz mislocalisation, since these proteins have previously been shown to be co-dependent on each other for recruitment to the junctions (Harris and Peifer, 2004). Baz staining in *lkb1* clones exhibited a number of defects, in addition to frequent mislocalisation from the AJ.

In wildtype PRCs, Arm and Baz expression overlap; in *lkb1* clones, these signals could be resolved from each other, with Arm frequently localising at a position basal to Baz. Interestingly, as well as a reduction at the junctions, Baz could also sometimes be seen to increase dramatically just apically to Arm, and this increase was always coincident with an apparent increase in Arm

protein. In addition, it was sometimes possible to observe Baz puncta, that were positioned basal to Arm and Baz expression in their normal position at the junctions. As with DaPKC, morphological defects apparent in the rhabdomeres and PRC morphology accompanied Baz mislocalisation [Fig. 4.13].

I next examined older pupal retinas at 55% pd, and noted that Baz could sometimes be seen to localise across the basal membrane of PRCs, and in addition, when separate ommatidial units appeared to fuse, Baz expression occasionally appeared to bridge between these ommatidia, seemingly connecting the separate PRCs [Fig. 4.13]. Taken together, the above data strongly suggests that Baz localisation in the developing PRC is dependent on Lkb1.

Technical difficulties precluded the examination of Par-6, since despite repeated efforts, and using several Par-6 antibodies, I was not able to get a Par-6 signal in pupal retinas. However, since the localisation of these proteins are co-dependent on each other, I would predict that Par-6 will show similar mislocalisation defects.

I have shown that members of the Par complex exhibit severe mislocalisation defects in the absence of *lkb1*; as well as reduced accumulation, DaPKC and Baz frequently show an expansion of staining in mutant PRCs. In addition, Baz is occasionally seen displaced basally to Arm, and is sometimes increased in accumulation at the adherens junctions. I have also shown that the defects in mislocalisation of these 2 proteins consistently accompany the morphogenetic defects seen in these *lkb1* clones.

Taken together, the above data indicate firstly, that Lkb1 is involved in regulating the localisation of these proteins. Secondly, since these defects consistently occur in tandem with morphogenetic defects, that mislocalisation of DaPKC, Arm, and Baz may be among the principal cues for the depolarisation of *lkb1* mutant PRCs. Finally, I speculate that Lkb1 may therefore be involved in directly modulating the localisation, or activity of these polarity determinants.

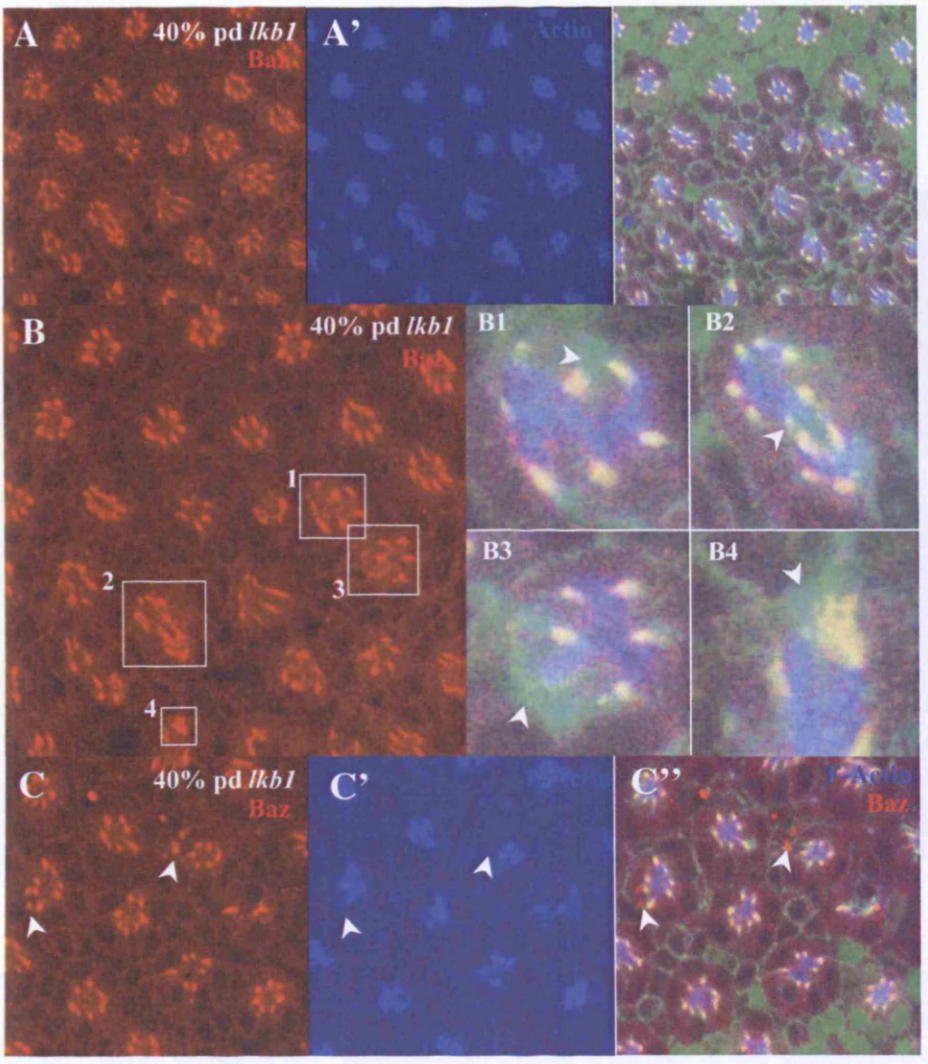



**Fig. 4-13 Loss of *lkb1* leads to a change in the distribution of Bazooka.** GFP marks wildtype tissue in all panels. F-actin is in blue. (A-C) Baz (red) is mislocalised and appears to be altered in levels in *lkb1* clones (white arrowhead). Baz is normally localised to the junctions and co-localises with the junctional marker, Arm (green). (B1) Baz localises apically to Arm (B2) *lkb1* ommatidia appear to be fused, and Baz expression appears to be expanded (B3) Baz appears to be decreased in levels, despite s significant upregulation of Arm at the junctions. (B4) Baz shows an upregulation in expression, as well as delocalisation from Arm expression. Arm frequently shows an expansion of expression to the basal domain. (C) Aberrant Baz expression can also be observed as puncta distinct from the normal positioning of Baz at the AJs (white arrowheads).

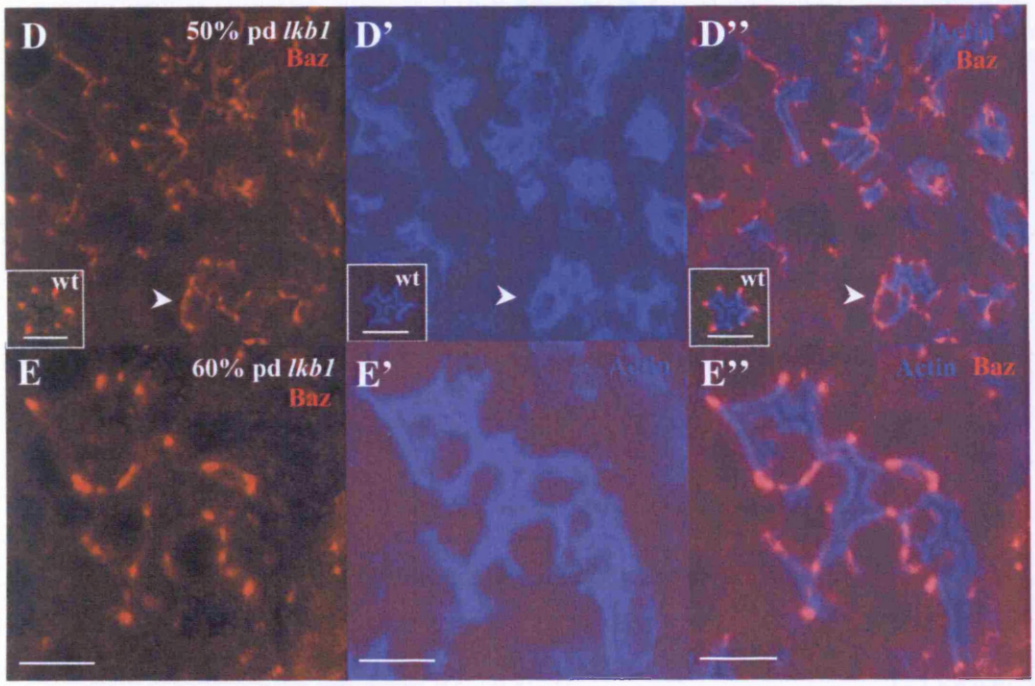



**Fig. 4-13 (cont) Loss of *lkb1* leads to a change in the distribution of Bazooka** (D-E'') *lkb1* clones in 50 and 60% pd retinas show gross defects in PRC and rhabdomere morphology; aberrant Baz expression frequently accompany these defects, e.g. mislocalisation of Baz along the apparent basal membrane (white arrowhead D). Baz mislocalisation persists in 60% pd clones. Scale bars represent 5μm.

## 4.9 Loss of *lkb1* can lead to the development of abnormal membrane domains at the lateral and junctional interface

Since the apical and junctional markers studied thus far show an apparent spread of staining towards the basal domain, I decided to examine the distribution of the junctional marker Arm with respect to a basal marker. Apical proteins appear to maintain apical localisation, showing some overlap with the junctional marker Arm, but largely remaining on the apical side of the PRCs. Arm shows a greater and more frequent spread towards the basal domain, indicating that there may be some overlap of membrane domains, or alternatively, that apical and junctional markers are expanding at the expense of the basal domain.

Na+/K+-ATPase is a sodium potassium pump that localises to the basolateral membranes in PRCs (Lebovitz et al., 1989; Sun et al., 1998). In wildtype PRCs, Na+/K+-ATPase is present on the basal and lateral membranes, with expression on the lateral membrane terminating just basal to the AJ.

A co-stain of *lkb1* clones with Arm and Na+/K+-ATPase showed significant overlap at the lateral membrane, suggesting that these PRCs have lost distinct lateral membrane identity [Fig. 4.14]. In addition, Na+/K+-ATPase also occasionally appeared increased in intensity at the lateral membrane.

An intriguing possibility is that the large cell bodies observed in the semi-thin sections of *lkb1* adult clones may be due to the defective localisation

of Na+/K+-ATPase. Na+/K+-ATPase is involved in the maintenance of appropriate ionic concentration within the cell, and thus regulates the volume of the cell. Mislocalisation of Na+/K+-ATPase from the basal membrane, or a compromise in basal membrane identity may lead to an imbalance of ionic concentrations within the cell, and lead to defective osmosis, thus producing over sized cells.

The above data provides further compelling evidence that Lkb1 is involved in the development of distinct membrane domains, the segregation of polar molecules, and the restriction of polarised components to their appropriate domains.

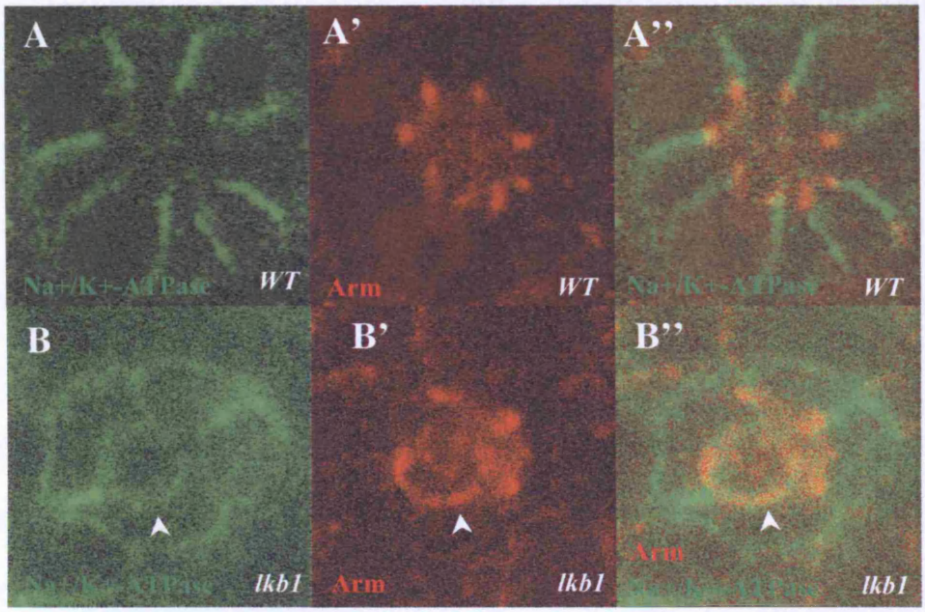



**Fig. 4-14 Junctional and basal markers overlap in *lkb1* clones.** GFP marks wildtype tissue. (A, A') Wildtype 40% pd ommatidia. Na+/K+-ATPase (green) marks the basolateral membranes, Arm (red) marks the adherens junctions. Arm and Na+/K+-ATPase are normally distinct in expression patterns (B, B') *lkb1* ommatidium shows an overlap of junctional and basolateral markers (white arrowhead) (experiment by Nancy Amin).

## 4.10 Cell polarity in *lkb1 4B1-11* clones in the pupal retina

Older clones of *lkb1 4A4-2* are too severely disrupted to examine the different membrane domains of mutant PRCs closely. Since clones deficient for *lkb1 4B1-11* show similar defects in the mislocalisation of the proteins examined above, but with less severity than is observed in *lkb1 4A4-2* clones, I decided to look at polarity defects in *lkb1 4B1-11* clones. PRCs that are mutant for *lkb1 4B1-11* show a greater degree of variability, from no defects to very severe defects in morphology. Other aspects of the *lkb1* phenotype, such as the fusion of 2 ommatidia, missing PRCs, or the basal spread of apical components such as Sdt, Crb, DaPKC etc were also observed [Fig. 4.15]. In order to follow the development of individual membrane domains, I looked at 55% pd retinas, and examined ommatidia that displayed the phenotype with moderate severity i.e. where defects in the localisation of several polarity proteins were apparent, but the morphology of the cells and the assembly of the rhabdomeres of a single ommatidium with respect to each other were largely intact.

As well as showing a basal spreading of Crb, cells with extreme basal spreading also displayed a 'rounding' up phenotype, whereby the membrane, at a single confocal plane, appears to be 'pinched' at the beginning of the basal membrane of the cell. Actin and Arm mark the cell outlines, and thus revealed this apparent constriction of the membrane at the basal region of the cell. In addition, single *lkb1* PRCs sometimes possessed more than one apical domain, highlighted by actin; or 3 sub apical membrane domains, as marked by Crb. I

also occasionally observed PRCs that stained exclusively with Actin and Crb, and lacked an apparent basal domain [Fig. 4.15].

The PRC is a thin and elongated cell that extends in the proximo-distal axis. Mutant PRCs that are buckled along the proximo-distal axis could lead to the illusion of PRCs that apparently possess more than the usual number of membrane domains. Additionally, obtuse angles produced by this type of contortion of the PRC may result in cross section images that show an apparent loss of the basal domain.

However, longitudinal images of *lkb1* PRCs do not indicate such severe defects in conformation [Fig. 4.9], suggesting that these defects may arise from the development of abnormal membrane domains.

At 50% pd, the depth of the retina is approximately 30 μm, and the rhabdomeral depth is approximately 20 μm (Longley and Ready, 1995). To try and determine whether this apparent defect in PRC morphology was due to conformational defects along the proximo-distal length of the PRC, I took z-series confocal images of 50% pd retinas containing *lkb1 4A4-2* clones at depths of 2 μm. Examining sections at the distal region of the retina near the cone cell roof, mid-retina, and at the proximal region of the retina, near the cone cell plate, revealed that the constriction of the basal membrane, just basal to the junctional markers Arm and DE-cad, persisted throughout the proximo-distal length of the PRC. This suggests that the phenotype may not be due to conformational defects in the proximo-distal axis [Fig. 4.16]. Further analysis of this phenotype could be to generate a 3D construction of a single PRC displaying this defect to more accurately assess the morphology of the PRC in the proximo-distal axis.

Further work is required to explain this phenotype, however this data does provides further evidence that Lkb1 functions to restrict the expression of polarised molecules to the appropriate domains, since in the absence of *lkb1* normally segregated markers overlap; apical and sub-apical markers (e.g. actin and Crb), sub-apical and junctional markers (Crb and Arm), apical and junctional markers (actin and Arm), and junctional and basal markers (Arm and Na+/K+-ATPase), indicating a loss of distinct membrane identity in *lkb1* mutant PRCs. Additionally, in the absence of *lkb1*, PRCs may appear to develop abnormal membrane domains, sometimes resulting in apparent extra apical or sub-apical domains.

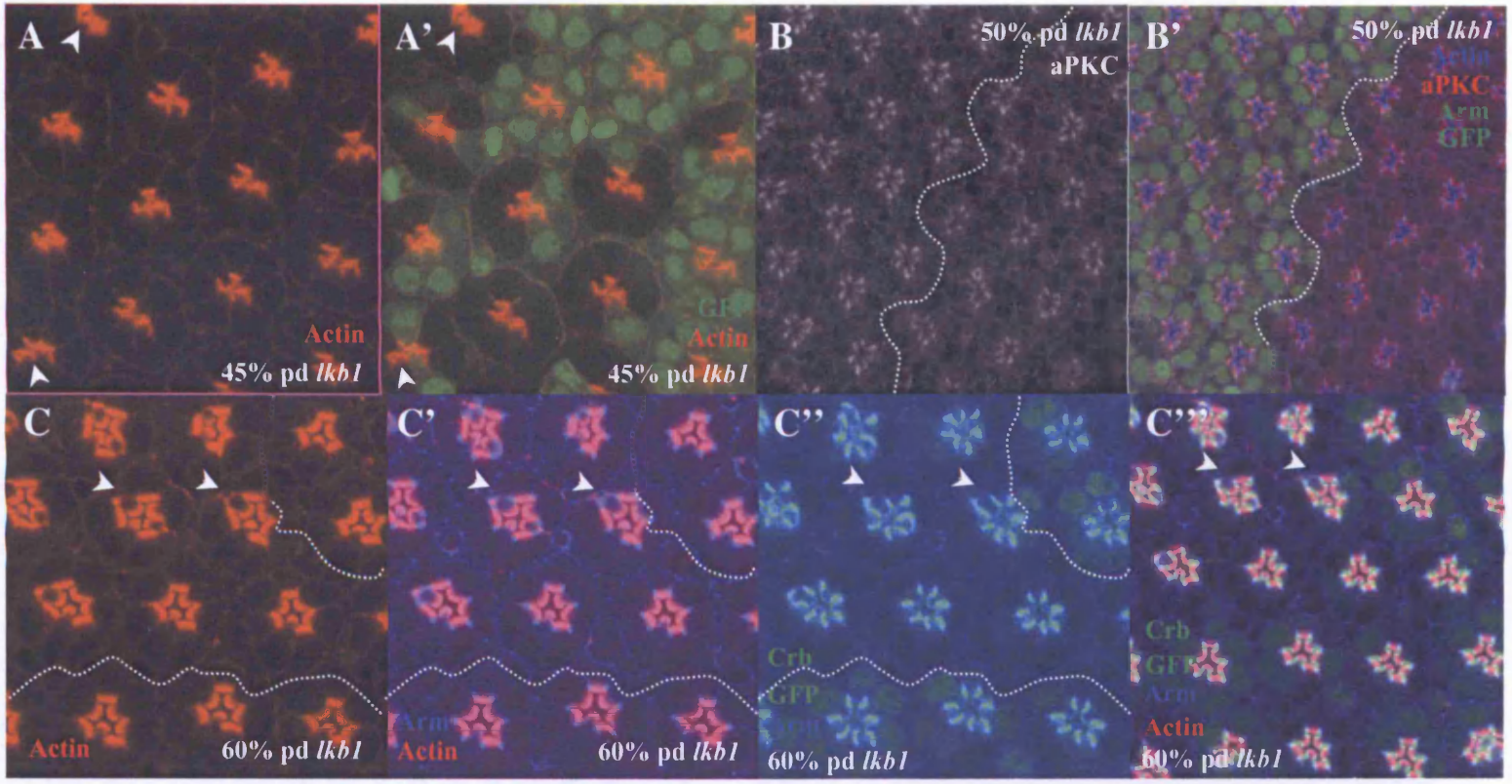



**Fig. 4-15 Polarity defects in *lkb1 4B1-11* clones.** *lkb1 4B1-11* clones are marked by the absence of GFP, and are to the right of the dashed lines (B,B') or within them (C-C''). (A) 45% pd *lkb1 4B1-11* clones show defects in rhabdomere morphology (actin; red) (white arrowheads). Older retinas show mislocalisation and a subtle decrease in the levels of DaPKC (B) and exhibit basal spreading and mislocalisation of Arm and Crb (C) (white arrowheads). *lkb1 4B1-11* ommatidia often show abnormal PRC and rhabdomere morphology, as well as missing PRCs. The defects are milder than that observed in *lkb1 4A4-2* clones.

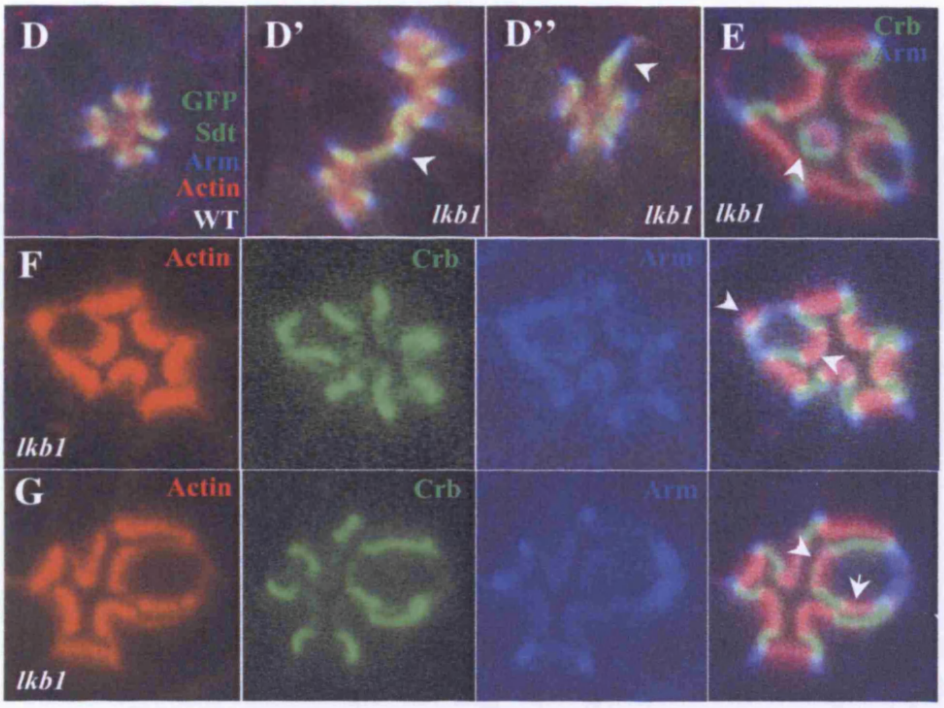



**Fig. 4-15 (cont) Polarity defects in *lkb1 4B1-11* clones** Sdt (green) (D-D'') is similarly mislocalised in *lkb1 4B1-11* mutant ommatidia (white arrowheads). (E) *lkb1 4B1-11* ommatidia occasionally show a rounding up of the R7 PRC (white arrowhead). Basal domains are absent from this structure, which stains only for apical and subapical markers. (F,G) *lkb1 4B1-11* ommatidia stained with markers against the different domains: apical domain (Actin is in red), subapical domain (Crb is in green), and junctional marker (Arm is in blue) revealed the presence of abnormal membrane domains within individual PRCs. Extra membrane domains are sometimes observed e.g 3 subapical domains, 2 apical domains (white arrowheads).

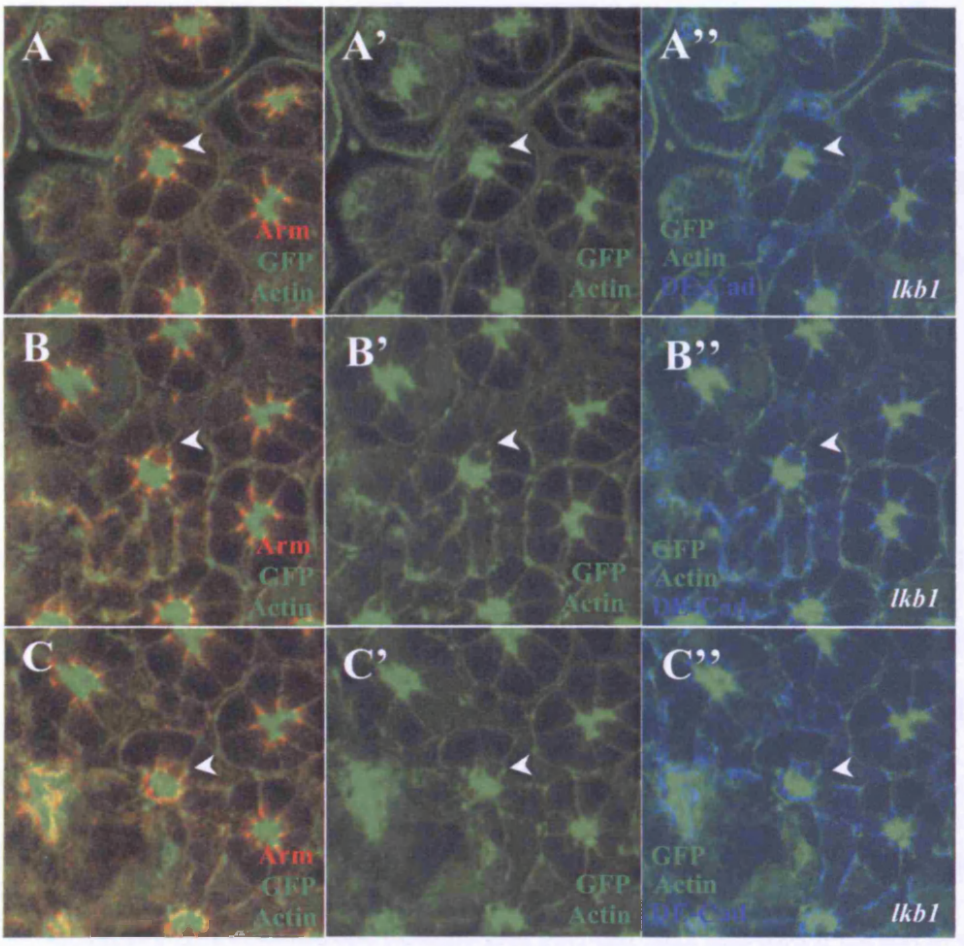



**Fig. 4-16 The expansion of apical and junctional markers persist throughout the proximo-distal length of *lkb1* mutant PRCs.** GFP marks wildtype tissue. Expansion of junctional markers Arm and DE-Cad (A, B, C) and the apparent constriction of the PRC cell at the basal domain (A', B', C') (marked by Actin, green) persists throughout the proximo-distal length of the PRC in *lkb1* clones in 50% pd retinas (white arrowhead). (A, A', A'') The distal region of the pupal retina, below the cone cell roof. (B, B', B'') Mid retina (C, C', C'') A proximal section of the retina, above the cone cell plate.

## 4.11 Lkb1 does not regulate the absolute levels of polarity determinants in the retina

Immuno-fluorescence data shows that the polarity determinants studied thus far are mislocalised in *lkb1* mutant retinas. Since there also occasionally appears to be an apparent increase or decrease of these proteins in *lkb1* PRCs, I decided to examine the relative abundance of these proteins in retinas that were completely mutant for *lkb1 versus* wildtype retinas.

I did this by using the previously characterised (section 4.1.1) *eyflp; minute 82BFRT* stock to create pupal retinas that were >95% mutant for Lkb1. 50% pd were the youngest retinas I could be certain were mutant, since I relied on the appearance of pigment at 50% pd as an indicator for the presence of *lkb1* clones. Hence, using the protocol described in Materials and Methods, I looked at western blots of 50% pd retinas, using β-tubulin as a loading control.

Contrary to expectation, neither DaPKC nor Baz showed a difference in protein abundance. Similarly, a change in protein levels was not observed in the Par complex partner Par-6, or SAR complex component Sdt [Fig. 4.17]. Thus, this data indicates that these proteins are mislocalised, but not reduced in protein levels. A decrease in the accumulation of DaPKC and Baz at the sub-apical and junctional membranes respectively does not reflect a decrease in protein levels, and instead, suggests a dispersion of protein throughout the PRC. Therefore, this data suggests that Lkb1 is required for the correct

localisation of polarity determinants, but is not involved in regulating the absolute levels of these proteins.

## 4.12 Lkb1 can phosphorylate polarity determinants *in vitro*

In order to test if the localisation of the polarity determinants described above may be regulated through Lkb1 mediated phosphorylation, I carried out an *in vitro* kinase assay using recombinant polarity proteins. Recombinant LKB1 was obtained from Cell Signalling Technologies, and is in a complex with its co-activators STRADα and mo25α that have been shown to be required for its activation (Baas et al., 2003; Boudeau et al., 2003; Hawley et al., 2003).

Previous reports have established that the localisation of polarity proteins can be regulated by phosphorylation events. In fact, DaPKC employs this method extensively to regulate the localisation of the septate junction complex, the SAR complex, and the basolateral protein Par-1 (Hurov et al., 2004; Hutterer et al., 2004; Sotillos et al., 2004). In addition to proteins being restricted to their domains by these interactions, phosphorylation events also act to modulate interactions with other proteins and association with the membrane e.g. Par-1, when phosphorylated by aPKC is inactivated and unable to associate with the membrane.

I therefore decided to look at a number of candidate polarity proteins to determine if any of these proteins which show a defect in localisation in *lkb1* clones could be potential substrates. Recombinant proteins of Crbi-GST (intracellular domain); full length aPKC-GST; Patj-MBP; Baz-GST; and Par-6-

MBP were exposed to the kinase active recombinant LKB1/STRADα/mo25α complex under the conditions described in Materials and Methods.

Baz-GST, DPATJ-MBP and Par-6-MBP could be phosphorylated by recombinant LKB1, whereas Crbi-GST and aPKC-GST were not phosphorylated in this context [Fig. 4.18]. In order to test if the phosphorylation of PATJ and Par-6 was specific to the proteins and not to the MBP tag, a colleague and I, using Factor Xa, cleaved the MBP tag from recombinant PATJ-MBP and repeated the kinase experiment. We found that PATJ was phosphorylated, while the 19kDa MBP tag was not, confirming specificity of LKB1 mediated phosphorylation to PATJ and Par-6 proteins.

Thus further exploration of Baz, Arm, DPATJ and Par-6 as candidate substrates of Lkb1, and the characterisation of potential regulatory sites may yield some insight into the mechanism by which Lkb1 might regulate the localisation of polarity determinants.

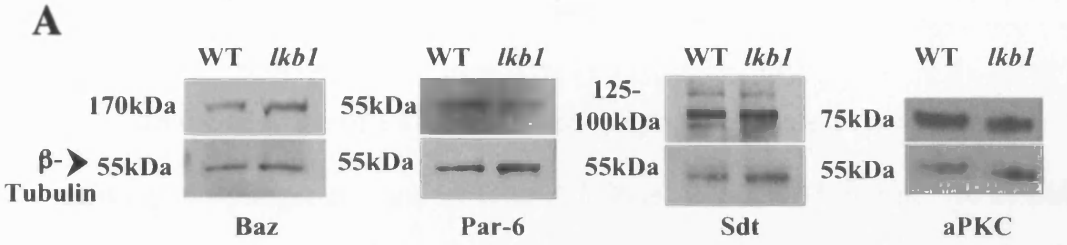



**Fig. 4-17 Protein levels of polarity proteins remain unchanged in *lkb1* mutant retinas.** (A) Western analysis of polarity determinants in 50% pd wildtype and *lkb1 4A4-2* mutant pupal retinas showed that the abundance of these proteins remain unchanged in *lkb1* mutant retinas. β-tubulin was used as a loading control.

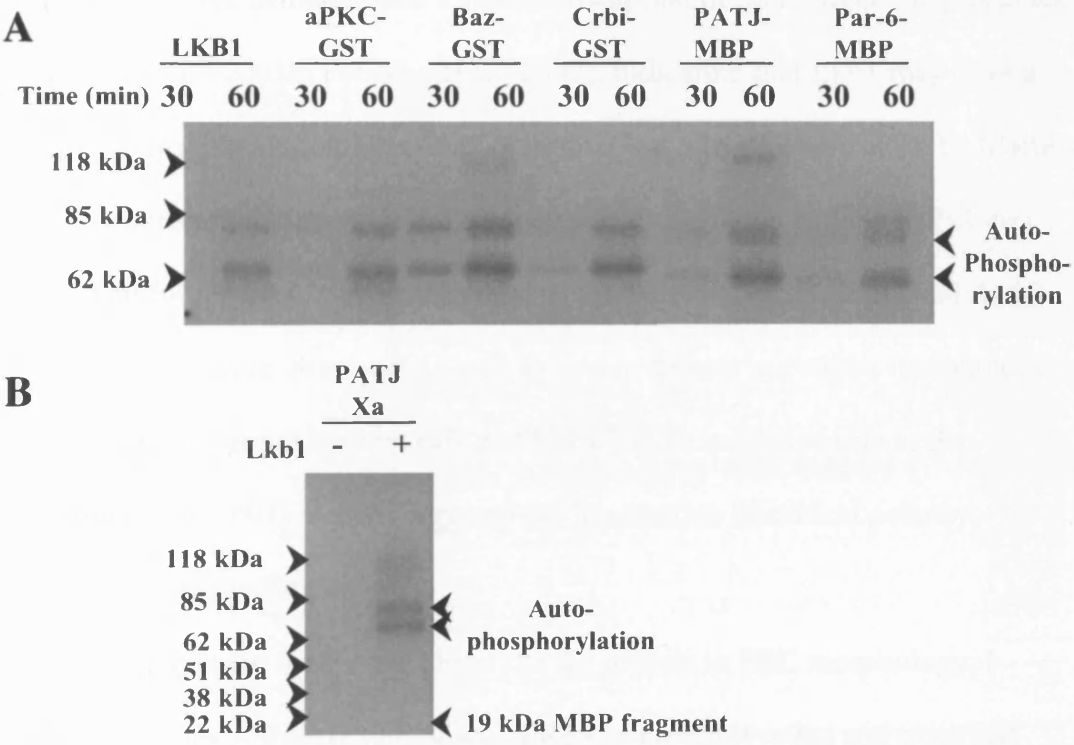



**Fig. 4-18 Lkb1 can phosphorylate polarity determinants *in vitro*.** (A) Recombinant LKB1/STRADα/Mo25α can phosphorylate recombinant tagged proteins Par-6-MBP, PATJ-MBP, and Baz-GST in an *in vitro* kinase assay. Crbi-GST and aPKC-GST were not phosphorylated by LKB1. Arrowheads on the right mark autophosphorylated LKB1, and molecular weight markers are indicated on the left (B) Cleavage of the MBP tag from Patj shows that phosphorylation is specific to the PATJ protein and not the MBP tag.

## 4.13 Discussion and conclusions

In the absence of Lkb1, clones in the pupal and adult retina display striking morphogenetic and structural defects. While *lkb1* mosaic eye clones show a variable penetrance of phenotype, these defects appear with high frequency in *lkb1* clones. Previous work exploring the mutant phenotypes of the apical junctional complex members, the Par complex and the SAR complex, have demonstrated a similar disruption of morphogenetic processes (Hong et al., 2003b; Pellikka et al., 2002), indicating that Lkb1 may have a parallel role in regulating epithelial morphology. In addition, work by Martin *et al* demonstrated that epithelial polarity was disrupted in follicle cell clones mutant for *lkb1* (Martin and St Johnston, 2003); defects in aPKC and Arm localisation were observed, as well as severe defects in cellular morphology. The data presented here reveals that Lkb1 has an extensive role in the regulation of PRC morphology and the localisation of critical polarity determinants in PRCs.

In order to look more closely at the defects in PRC morphology, I examined the ultrastructure of *lkb1* PRCs in the adult retina and observed defects in the stalk membrane, AJs and base membrane of the rhabdomere. Defects such as these are suggestive of disruptions in cellular polarity, and have been reported in the characterisation of other polarity mutants. In order to test this and to characterise the polarity status of *lkb1* tissue, I set out to examine these morphogenetic defects using immuno-histochemical and biochemical assays.

*Lkb1* ommatidia frequently have missing PRCs. To exclude the possibility that missing PRCs may be a result of defects in cell fate processes, I looked at a number of neuronal markers in the larval eye disc. I found that all PRCs were correctly specified in *lkb1* clones, demonstrating that loss of PRCs was due to cell death rather than cell fate defects, and that Lkb1 therefore does not affect the initial commitment to a PRC fate, but instead has a role in the maintenance and/or morphogenesis of PRCs.

I did however find mild defects in PCP, as revealed by Bar staining of PRCs 1 and 6. It is recognised that the loss of cell polarity can sometimes lead to the loss of planar cell polarity, as cells need PCP determinants to be deposited into specific domains of the cell in order to complete the patterning process (Wasserscheid et al., 2007). However, since later experiments examining cell polarity in the larval eye disc did not identify defects in cell polarity, this is unlikely to be a cause of PCP defects in *lkb1* clones. Instead, it is possible that Lkb1 may have a limited role in patterning in the larval eye disc.

The rhabdomere is a post larvae specific structure, which is assembled during pupal development at the apical most region of the cell; defects in the shape and structure of the rhabdomere indicate defects in apico-basal polarity. In order to investigate a role for Lkb1 in regulating epithelial polarity, I began by examining its localisation in the larval disc. Lkb1 is also a Par protein and the Par proteins have been shown to have roles in the control of cell polarity in many organisms (Macara, 2004).

Both in wildtype eye discs and discs overexpressing Lkb1, I observed a faint cortical localisation of Lkb1 in ommatidial preclusters; this is in line with

observations of Lkb1 localisation in other model systems, including *Drosophila*, which report the ubiquitous cortical localisation of Lkb1 (Martin and St Johnston, 2003). Since Lkb1 has been shown to associate with the cortex in mammalian cells, as well as in this study, it is possible that Lkb1 can act as part of a conserved phosphorylation/regulatory cascade to regulate the activities of cortically localised polarity complexes.

I next examined the localisation of polarity markers in order to determine whether defects in adult PRC morphology could be attributed to an initial disruption of cell polarity in the larval eye disc. I looked at aPKC, Bazooka and Arm; all were localised normally in *lkb1* clones in the eye disc. Taken together, data from the larval eye disc suggest that the loss of PRCs and morphogenetic defects observed in clones in the adult eye occur during pupal development.

Potential reasons for this are, firstly, the perdurance of Lkb1 protein. This is possible, given that the perdurance of Lkb1 has been previously demonstrated in *Drosophila (Martin and St Johnston, 2003)*, however, data from the larval disc showing increased apoptosis and PCP defects in *lkb1* clones suggest that perdurance may not be a factor in the larval eye disc. Secondly, shortly after pupation, ommatidia preclusters undergo a dramatic reorganisation and elaboration of their morphology; Lkb1 may therefore have a necessary role in the remodelling of cell polarity that occurs later during pupal morphogenesis.

In order to identify when morphogenetic defects in *lkb1* tissue were occurring, I began by examining rhabdomere structure throughout pupal development. Since the *4A4-2* allele is a deletion allele that completely lacks Lkb1 protein expression, and can be almost completely rescued by the

expression of Lkb1 under its genomic promoter, I primarily used this allele in the characterisation of morphogenetic defects.

*lkb1* mosaic retinas were stained for actin, which highlights the rhabdomere and the cell outlines. Defects become apparent at 10% pd, when ommatidia, viewed from the side, appeared to be dropping out of the plane of the retinal epithelium. Images taken at cross section at 20% and 30% pd further revealed defects in the morphology of the developing ommatidia in *lkb1* clones. Cells were often irregularly shaped, I occasionally observed a fusion of 2 PRC clusters, and the mutant tissue was generally disorganised.

At 40% pd, it was apparent that by this stage, PRCs begin to be lost from ommatidia, and at 55% pd, *lkb1* ommatidia and rhabdomeres are grossly misshapen, and continue to show fusion between multiple ommatidia.

Data from the *4B1-11* allele revealed similar defects, but were less severe. The above data demonstrated that defects in PRC morphology occur soon after pupation, and persist throughout development, leading to severe defects, including the occasional fusion of ommatidia that lead to a loss of distinct ommatidial identity.

These defects were reminiscent of morphogenetic defects seen in mutants of the SAR complex, and in addition data from TEM analysis of *lkb1* clones revealed phenotypic similarities to aspects of *crb* and *sdt* mutants. Keeping this in mind, I began to characterise the development of polarity in *lkb1* clones by first examining the subcellular localisation of Crb, Sdt and DPATJ. I carried out an analysis of these proteins throughout the different stages of pupal development and determined that all members of the SAR complex were frequently mislocalised in *lkb1* clones.

Crb is restricted to the apical domain, co-localising with the rhabdomere, until approx 50% pd, when Crb can be found at a sub apical position, just basal to the apical domain. In *lkb1* clones, Crb is often mislocalised to the basal domain, and spots of Crb staining, distinct from the main body of Crb protein at the apical domain can often be seen at the basolateral portion of the PRC. Despite this mislocalisation of Crb, however, defects in Crb do not always accompany morphogenetic defects, indicating that the mislocalisation of Crb is not a necessary event for the morphogenetic defects to occur.

Sdt and DPATJ showed similar defects in mislocalisation, but again, were not required to be mislocalised for morphogenetic defects to take place.

Loss of *lkb1* clones in the retina share many similarities to the *crb* and *sdt* phenotypes; as well as the more general polarity related phenotypes such as aberrant rhabdomere morphogenesis, loss of Crb leads to a reduction in stalk length and a disruption of the ZA. *lkb1* retinas also display an occasional defect in stalk membrane length. Similarly, the overextension of the base membrane of *sdt* mutant rhabdomeres is also seen in *lkb1* retinas. This suggests that although defects in SAR complex localisation appear to be secondary effects, that the mislocalisation of these proteins is significant and may contribute to the *lkb1* polarity phenotype.

Studies of polarity determinants have established that the SAR and Par complex proteins are mutually dependent on each other for their localisation e.g. Crb can bind Par-6 (Kempkens et al., 2006). Since proteins of the SAR complex show mislocalisation, I expected to see similar defects in proteins of the Par complex: DaPKC, Baz, and Par-6. In fact, in addition to mislocalisation of DaPKC and Baz, I also observed accumulation defects, so that in some

instances, DaPKC or Baz appeared to be absent from their normal localisation on the membrane. Mislocalisation of DaPKC and Baz also appeared to be more severe, often spreading throughout the PRC. Baz, which normally localises to the AJ and co-localises with Arm, could sometimes be resolved as separate spots from Arm; since Baz and Arm are both crucial components of the AJ, this, and general defects in the localisation of Arm suggest disruption of the AJs in *lkb1* PRCs.

Critically, defects in the morphology of the PRCs were consistently accompanied by defects in DaPKC and Baz, strongly suggesting that the mislocalisation of these components are critical events for the morphogenetic defects observed in *lkb1* PRCs.

In addition to the immuno-fluorescence data from the pupal retina, a genetic interaction experiment suggested that DaPKC and Lkb1 may function in the same pathway. The main aspect of the *lkb1* phenotype that was enhanced were the morphogenetic defects in the rhabdomeres, indicating that DaPKC and Lkb1 may function synergistically in a polarity pathway. Epistatic analysis place DaPKC downstream of Lkb1, an observation supported by immuno-histochemical data showing misregulation of DaPKC in the absence of Lkb1. However, Lkb1 could not phosphorylate full length DaPKC in an *in vitro* context, suggesting either that regulation of DaPKC by Lkb1 is not direct, or that the *in vitro* kinase assay could not accurately replicate *in vivo* conditions necessary for DaPKC phosphorylation.

Since reducing the gene dosage of DaPKC by 50% results in an enhancement of the *lkb1* phenotype, additional experimental opportunities present themselves. One informative experiment would be to see if

overexpressing DaPKC could rescue aspects of the *lkb1* phenotype. Questions that remain unanswered include: whether DaPKC is still active despite the defects in accumulation, and how are downstream targets of DaPKC affected. Since DaPKC operates as part of a mutual exclusion mechanism with other polarity determinants, loss of either DaPKC localisation or activity may precipitate further defects in the localisation of other polarity determinants e.g. Par-1, or the septate junction complex.

Strikingly, Arm, a major component of the adherens junctions frequently showed mislocalisation in *lkb1* clones, indicating that the mislocalisation of Arm may also be one of the primary, and/or causative defects in Lkb1 deficient retinas. This is discussed more fully in Chapter 5.

In order to determine whether the expansion of junctional marker expression reflected a physical expansion of the junctional domains at the expense of the basal domain, I examined the basal marker Na+/K+-ATPase, in conjunction with the junctional marker Arm. Co-localisation studies with Arm and actin showed that Na+/K+-ATPase overlapped with Arm in *lkb1* PRCs, indicating defects in membrane identity, and further suggesting a possible ectopic expansion of junctions along the lateral membrane. Thus, the loss of *lkb1* did not appear to favour an expansion of the junctional domain, but rather a loss of distinct membrane identity, and impairment of the mechanisms that normally serve to restrict polarised proteins to their respective domains.

Immuno-fluorescence data generated from *lkb1 4B1-11* clones also suggested the formation of abnormal membrane domains. In addition to the overlap of normally distinct markers that is also observed in *lkb1 4A4-2* clones, I frequently observed a surplus of membrane domains per PRC, e.g. 2 apical

domains, highlighted by actin, or >2 sub apical domains, marked by Crb. Despite their positioning in inappropriate regions of the cell, these ectopic domains appear to be very distinct from each other, with no overlap of polarity markers from different regions, suggesting an initial inappropriate specification of membrane domains, rather than a 'leakage' of cortical polarity proteins into other domains.

Furthermore, I also occasionally observed an apparent rounding up of the apical, sub apical and junctional domains of the PRC, so that there appeared to be a constriction of the membrane where the junctional markers terminated. These complex phenotypes are difficult to interpret; conformational changes in the PRC may lead to these defects, or alternatively, the assembly of the different domains in *lkb1* PRCs is indeed affected, and Lkb1 may therefore have a role in the correct specification of membrane domains, the maintenance of distinct membrane domains, and the restriction of polarity determinants to their appropriate regions. Further work to answer this question could include high-resolution 3D reconstruction of these ommatidia to examine the conformation and the positioning of membrane domains in these PRCs.

Taken together, these results indicate a cellular wide effect of the loss of *lkb1* on the localisation of multiple polarity determinants. Whether these severe defects in polarity and morphology arise from the misregulation of multiple polarity proteins, or a few key proteins remains to be determined.

Crucially, the mislocalisation of proteins that have key functions in the maintenance of polarity suggest the loss of central mechanisms for the generation and maintenance of polarity.

An interesting and related question posed by Bayraktar and colleagues in their exploration of Par-1 and its function in epithelial polarity, and one that is equally pertinent in this context was whether the mislocalisation of normally polarised determinants was due to a change in the properties of the membrane domains, or whether it is due to a mistargeting of vesicles to an inappropriate membrane domain. The polarised distribution of proteins is mediated by a variety of mechanisms, e.g. lateral exclusion mechanisms which restrict protein and protein activity to their relevant domains; positive recruitment processes e.g. the recruitment of apical factors by Baz during cellularisation; and the delivery of cytoplasmic vesicles to discrete membrane domains i.e. mediated by the exocyst complex.

Since the loss of *lkb1* leads to the mislocalisation of a number of polarity proteins that are involved in lateral exclusion and positive recruitment mechanisms, it is likely that these systems are disrupted in *lkb1* mutant clones. But what about transport mediated processes such as exocytosis? The exocyst is a multi-subunit protein complex that resides at specified areas on the membrane, and facilitates the delivery of cytoplasmic vesicles to plasma membrane domains by enabling secretory vesicle targeting and docking. Inhibition of exocyst function leads to a disruption of cell polarity (Beronja et al., 2005). Since the exocyst complex resides at sites of vesicle fusion, it may be possible that mislocalisation of the exocyst complex at the membrane can lead to mistargeting of polar complexes at the membrane. Additionally, the exocyst complex interacts with Arm, and is located at the AJs (Langevin et al., 2005) both of which show defects in *lkb1* clones, suggesting that the exocyst may therefore be compromised in function.

However, 2 points argue against exocyst function being affected in *lkb1* clones; firstly, these defects cannot be occurring primarily through compromised exocyst function, since Beronja *et al* showed that the inhibition of exocyst function by reduced function in Sec6 does not lead to the mislocalisation of Crb and DE-Cad, whereas these are mislocalised in *lkb1* PRCs (Beronja et al., 2005). Secondly, Beronja *et al* also showed that reduced function in Sec6 led to a failure of proteins to be apically transported to the rhabdomere. Polarity determinants such as Baz, DaPKC and Crb exhibit dynamic localisation throughout pupal development, and this is mediated by polarised transport. However there appears to be no defect in these directed movements in mid stage pupal retinas since Crb is still able to localise from the apical domain into the sub-apical domain in *lkb1* clones (>40%) and aPKC from the sub-apical domain into the apical rhabdomere in late pupal development (data not shown). This suggests that *lkb1* mediated control of epithelial polarity and the exocyst complex function in cell polarity may be separate and distinct processes.

Many polarity determinants have been shown to be regulated by phosphorylation events (Benton and St Johnston, 2003b; Hurov et al., 2004; Sotillos et al., 2004; Suzuki et al., 2004). Therefore, to test if Lkb1 could potentially regulate some of these polarity determinants by phosphorylation, I carried out an *in vitro* kinase assay, using the recombinant proteins DPATJ, aPKC, Bazooka, the intracellular domain of Crb, and Par-6. I found that Lkb1 could phosphorylate Bazooka, Par-6, and DPATJ, suggesting that these proteins contain motifs which can be phosphorylated by Lkb1, and that

therefore it is possible that these interactions may be observed in an *in vivo* context too.

Ongoing and further analysis of this data could include the identification of the specific sites on the recombinant proteins that are being phosphorylated. This is likely to involve the use of peptide mapping experiments, whereby overlapping peptides derived from the protein of interest are spotted onto a membrane and a kinase assay carried out. Longer-term goals will be to determine the phosphorylation status of these proteins *in vivo* in *lkb1* tissue, and *lkb1* overexpression tissue; this will also involve the generation of phospho-specific antibodies to these sites. A detailed analysis of the functional significance of these phosphorylation events will include mutation analysis and generation of UAS constructs with mutated phosphorylation sites to employ in rescue experiments.

One feature of the *lkb1* phenotype that was not explored in further detail was the PCP defect. I observed PCP defects in *lkb1* deficient clones both in the larval eye disc, and in the adult retina. In addition to A-P and D-V flips, I also observed misrotation defects. The mechanistic aspects of the ommatidial rotation process remain unknown, but DE-cad and DN-cad mutants have been shown to have misrotation defects - the rotation of PRC clusters within the plane of the epithelium require changes in cell-cell contacts (Klein and Mlodzik, 2005). This may suggest additional defects in retinal patterning that are brought about by the misregulation of junctional components in *lkb1* clones.

However, the specification of PCP is a complex process that requires input from many signalling pathways. As well as regulating PCP, these

pathways are also involved in other processes, including apoptosis (JNK)(Yamanaka et al., 2002), and proliferation (Wg) (Povelones et al., 2005). In addition, apical determinants aPKC and Dpatj, shown in this study to be mislocalised in *lkb1* clones, have been demonstrated to regulate PCP via a Fz dependent mechanism (Djiane et al., 2005). Hence PCP defects in *lkb1* mutants may be incidental to a deregulation of other processes that Lkb1 has a more direct role in regulating.

The data presented in this chapter suggest a required function of Lkb1 for the regulation of epithelial polarity in the eye. These results further delineate possible roles for Lkb1 during specific periods of development and morphogenesis; Lkb1 may have a limited role during eye disc patterning in the larvae, and a more pivotal role in the maintenance or reorganisation of cellular polarity during pupal development.

Further analysis of this reported role of Lkb1 in regulating polarity complexes will focus on uncovering the mechanism or mechanisms that Lkb1 normally employs to maintain distinct membrane domains. In particular epistasis experiments will be especially informative; since genetic interactions have shown that dominant interactions can be observed in the *lkb1* clonal assay system, this system can be used to further explore the effects of loss of *lkb1* on these polarity effectors.

# 5 Loss of Lkb1 leads to the misregulation of Armadillo, Par-1 and defects in adherens junction formation

Initially I had sought to characterise the polarity status of clones deficient for *lkb1* by looking at the localisation and distribution patterns of the apical and sub-apical polarity complex proteins. I used Arm antibody to mark the AJs, as a way of demarcating the polarised membranes of the cell, and to test whether the restriction of apical markers to the apical domain was compromised. I observed that Arm, a crucial component of the AJs, was frequently mislocalised in *lkb1* clones.

AJs are thought to act as a landmark for the polarised cell alongside other cues such as cytoskeletal and extra-cellular matrix (ECM) cues. In addition to acting as a physical landmark, AJs also act as a 'reservoir' for various factors involved in regulating polarity, such as members of the Par complex, or the exocyst complex (Beronja et al., 2005), and thus plays a significant role in the regulation of cell polarity.

In the following chapter, I present evidence that Lkb1 has a role in determining the correct localisation of Arm, that Arm is stabilised in the absence of Lkb1, and that Lkb1 regulates the formation of adherens junctions in the pupal retina. I further demonstrate that Lkb1 genetically interacts with Par-1, a polarity molecule involved in regulating epithelial polarity and the

microtubule cytoskeleton; and in the absence of Lkb1, Par-1 is stabilised at the basolateral membranes.

Additionally, I show that loss of Lkb1 can lead to a decrease in the inhibitory phosphorylation of Par-1, indicating a role for Lkb1 in the processing and regulation of Par-1.

Finally, I present immuno-fluorescence data that demonstrates that loss of *lkb1* in the early pupal retina gives rise to severe defects in cone cell morphology and configuration.

## 5.1 The junctional marker Armadillo is mislocalised in *lkb1* clones in the pupal retina

In order to examine the membrane structure of *lkb1* PRCs in pupal retinas, I used a number of markers for the different membrane domains; Arm was used to mark the AJs. I found that *lkb1* clones showed localisation defects in Arm which were apparent from 20% pupal development (pd). I examined the expression patterns of Arm in *lkb1* clones throughout pupal development, and found that Arm frequently showed expansion of expression, and was often increased in intensity around the junctions and generally throughout the cell in clones that are mid pupal development. Arm mislocalisation most frequently appeared as an expansion of Arm staining towards the basal domain [Fig. 5.1]. In *lkb1 4B1-11* clones mutant ommatidia often appeared as spider-like assemblies, with Arm showing an apparent spreading towards the basal domain, in tandem with what seemed to be a constriction of the membrane at

the basal domain [Fig. 4.16]. In addition, Arm also sometimes displayed a diffuse distribution throughout the cell.

These mislocalisation defects occurred from approx 20% pd, and accompanied every incidence of basal spreading by other apical factors, suggesting that defects in Arm localisation may be one of the primary defects in *lkb1* clones, and may be a precipitating factor for the mislocalisation of other proteins. Arm mislocalisation also persists in older retinas, until at least 80% pd [Fig. 5-1 and data not shown].

Immuno-fluorescence data strongly suggested that Lkb1 is required for the normal regulation of Arm localisation and protein levels, however, I did not observe defects in Arm earlier than 20% pd. One reason for this may be because the cells undergo a morphological change before this stage that precipitate the defects in Arm; a possible scenario given that the transition from larval to pupal development involves many morphogenetic changes. Alternatively, since perdurance of Lkb1 protein has been observed in germline clones in the oocyte (Martin and St Johnston, 2003), it may be that pre-clonal Lkb1 protein starts to degrade during this period, and that the loss of function phenotypes of Lkb1 are subsequently only apparent at this stage; however this is unlikely since loss of *lkb1* clones in the larval eye disc reveal defects in apoptosis and patterning. Finally, there may not be a requirement for Lkb1 mediated regulation of Arm, until approx 20% pd, when there may be a re-specification of cellular polarity (Longley and Ready, 1995).

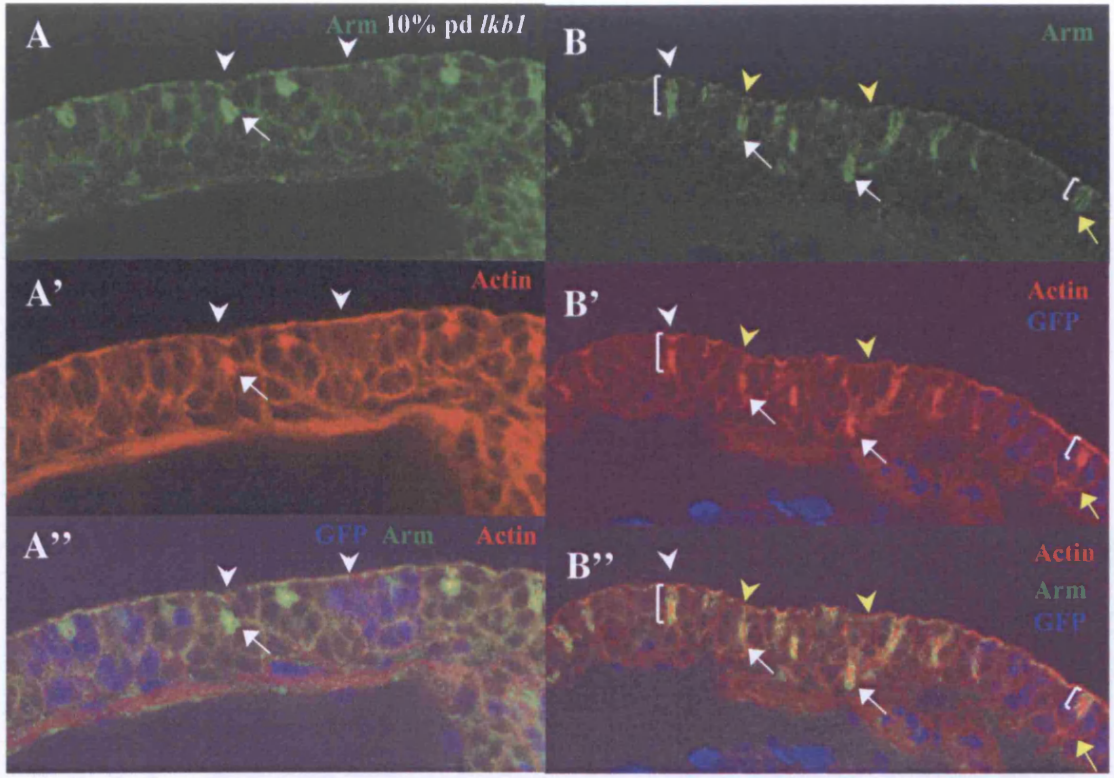



**Fig. 5-1 Loss of *lkb1* leads to a change in the distribution of Armadillo**. GFP (blue) marks the wildtype tissue. (A-A'') At 10% pd, mutant ommatidia appear to detach from the apex of the retinal epithelium (white arrow). Actin (red) shows a decrease in expression and Arm (green) shows normal distribution at the apical surface of the epithelium. (B-B'') At 20% pd, in addition to attachment defects, mutant ommatidia show further defects in ommatidial morphology; wildtype ommatidia are organised in a regular array and pattern (yellow arrow), while mutant ommatidia frequently appear distorted and elongated in shape (white scale bars). Ommatidia also appear at the base of the epithelium (white arrow), and actin is reduced in intensity at the apical surface (yellow arrowheads), with normal distribution seen only directly above the ommatidia which remain close to the apical surface (white arrowhead).

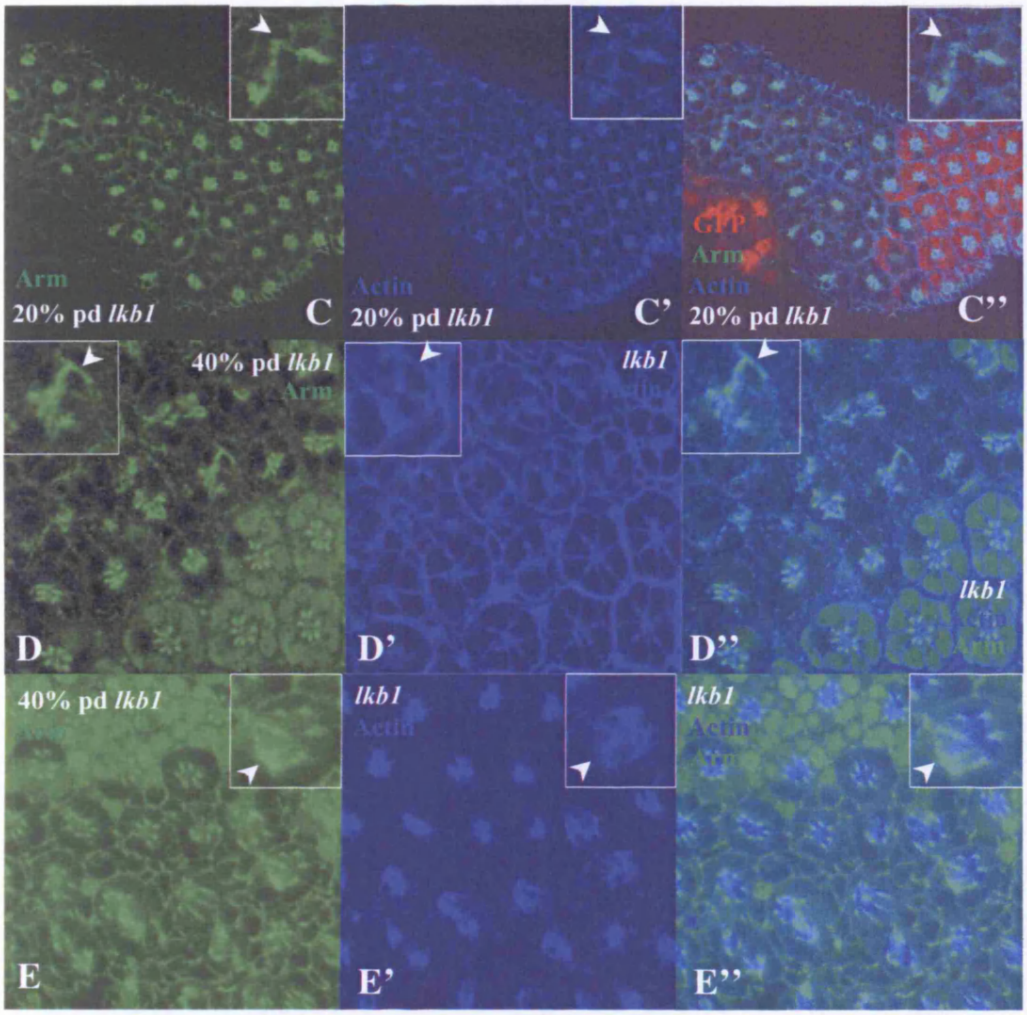



**Fig. 5-1 (cont) Loss of *lkb1* leads to a change in the distribution of Armadillo.** (C) Cross sections at 20% pd show defects in Arm localisation (green); Arm frequently mislocalises to the basal membrane (white arrowhead, inset). (D-E'') 40% pd *lkb1* clones. Arm frequently shows mislocalisation to the basal membrane (D-D'', inset), and upregulation (E-E'', inset).

## 5.2 Armadillo protein levels are post-transcriptionally stabilised in *lkb1* mutant tissue

Immuno-histochemical data from *lkb1* pupal retinas frequently revealed an apparent increase of Arm at the AJs, and occasionally throughout the cell body. To test whether this was a real increase in protein levels, I compared the levels of Arm protein in the western assay system previously established to examine the levels of polarity proteins. A western probed using an antibody for Arm that recognises the N-terminus, revealed that total levels of Arm were indeed increased two-fold [Fig 5-2].

This result demonstrated that in the absence of Lkb1, Arm is upregulated. In order to determine whether this increase was transcriptional or post-transcriptional, a colleague in the lab undertook a RT-PCR analysis of wildtype retinas vs *lkb1* mutant retinas, using primers designed against the exon boundary 4-5 of the Arm gene. RT-PCR results did not show a change in the levels of Arm transcript in *lkb1* mutant retinas compared to wildtype retinas, indicating that Arm transcript levels are not altered in *lkb1* mutants [Fig. 5-2]. This data provides evidence that the increase in Arm protein is not due to transcriptional upregulation but instead is a result of post-transcriptional stabilisation of Arm protein.

I repeated these experiments a number of times, using mutant retinas of both *lkb1* alleles. In my western assays I consistently found a reproducible, and clear increase in ubiquitous Arm, and these results were consistent with data

from immuno-histochemical experiments in *lkb1* clones that also indicated an excess of Arm protein. Since these experiments were conducted however, a colleague in the lab has attempted to reproduce this experiment. She has obtained results that suggest that the observed increase in Arm may be an artefact of the dissection protocol, and that in western sample preparations from *lkb1* mutants, an excess of basal lamina tissue may be contributing to the extra Arm protein. Currently efforts to establish whether Arm is upregulated in *lkb1* clones are ongoing. The following experiments were conducted on the premise that Arm is upregulated in the absence of Lkb1.

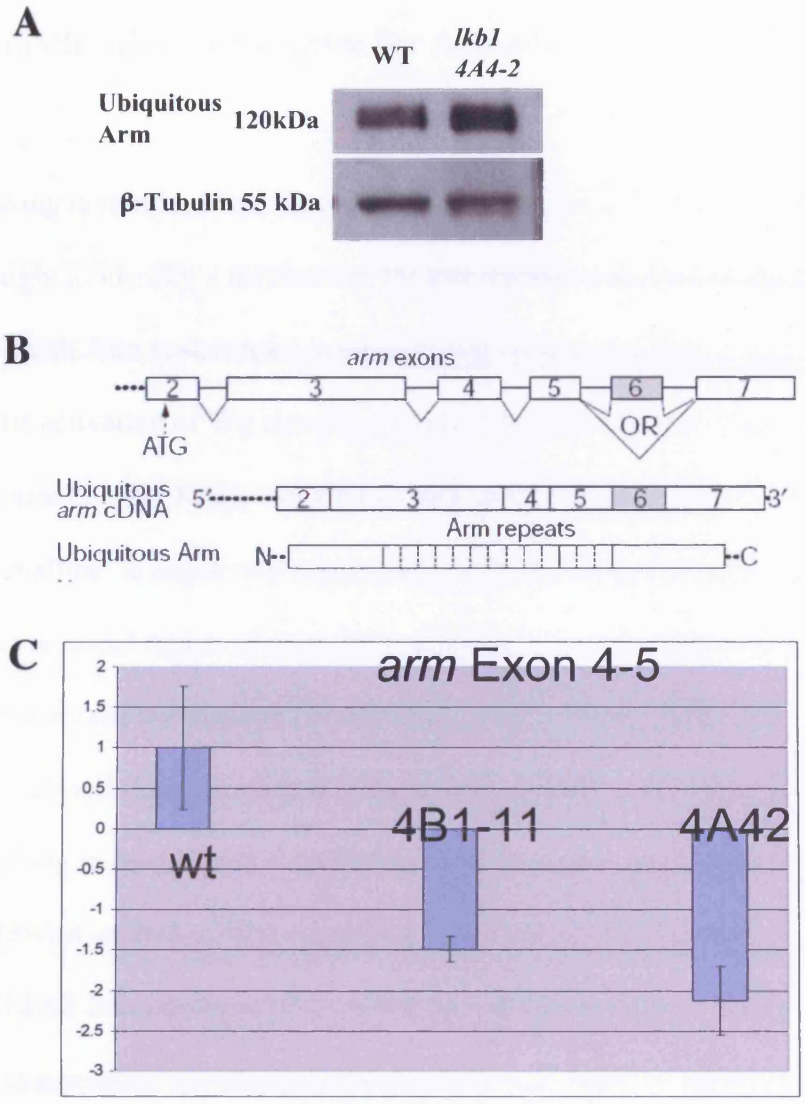



**Fig. 5-2 Armadillo is post-transcriptionally stabilised in *lkb1* mutant retinas.** (A) Western analysis of Arm in *lkb1 4A4-2* retinas show that Arm is upregulated in the absence of Lkb1. β-Tubulin is the loading control. (B) Schematic of the Arm gene. (C) RT-PCR results indicate that Arm transcript levels are not altered in *lkb1* mutants. Primers were designed against the exon boundary 4-5. The vertical is the fold change (experiment conducted by Nancy Amin).

### 5.2.1 Increased Wg signalling in *lkb1* clones is not a stabilisation mechanism for Armadillo

Having determined that Arm protein is stabilised in the absence of Lkb1, I next sought to identify a mechanism for this stabilisation. One of the primary ways in which Arm is stabilised is through Wg (Wnt) signalling (Liu et al., 2005). The activation of Wg signalling leads to the inhibition of glycogen synthase kinase 3 (GSK3β), and the cytosolic pool of Arm, which is competent in Wg signalling, is negatively regulated by GSK3β. GSK-3 phosphorylates the amino-terminal region of Arm, thus targeting it for ubiquitination and degradation by the proteosome (Aberle et al., 1997; Polakis, 2001).

I wondered if the increase in Arm protein could be due to an activation of Wg signalling in *lkb1* clones. Conflicting data has previously been reported for Lkb1 mediated control of Wnt signalling. Ossipova *et al* report that LKB1/XEEK1 acts upstream of β-catenin by regulating GSK3β, and is required to potentiate the Wnt signalling pathway. Similarly, Spicer *et al* demonstrated that LKB1 can potentiate the Wnt pathway by activating PAR1A, a positive regulator of the Wnt signalling. In contrast, Lin-Marq *et al* report that LKB1 mutant cell lines were unable to activate GSK3β, leading to an upregulation of Wnt signalling in the absence of LKB1 (Lin-Marq et al., 2005; Ossipova et al., 2003; Spicer et al., 2003).

In order to analyse the status of Wg signalling in *lkb1* mutant clones, I began by examining the external morphology of adult retinas mutant for *lkb1*.

Two studies by Cadigan *et al* demonstrated that a readout of upregulated Wg signalling in the adult eye was the loss of interommatidial bristles (Cadigan et al., 2002; Cadigan and Nusse, 1996). Activated Wg signalling blocks the expression of proneural gene expression, which is required in the developing eye for the specification of the interommatidial bristles, by repressing Daughterless expression in the proneural clusters.

SEM analysis of adult retinas wholly mutant for *lkb1* revealed that despite defects in the number and placement of bristles, there was no detectable loss of interommatidial bristles. Instead, bristles displayed defects in orientation, length and were generally disorganised [Fig. 5.3].

However, since the selection of the bristle progenitors, the sensory organ precursors (SOPs), takes place after the onset of pupation, the activation of Wg signalling after this period cannot be detected using this method. This data therefore presents evidence that Wg signalling is not hyperactivated during the selection of the SOPs, which occurs soon after the onset of pupation.

Since the results of this experiment were limited to a specific period, I then looked at other ways of assessing Wg signalling in *lkb1* mutant retinas. There are 3 biochemical readouts for Wg signalling that are frequently examined to assess the activation status of this pathway: 1. The stabilisation of Arm (Matsubayashi et al., 2004), 2. Hyper-phosphorylation of Dishevelled (Dsh) (Matsubayashi et al., 2004), and 3. Upregulation of Wg target genes. Having detected the stabilisation of Arm in *lkb1* retinas, I next looked at the activation status of Dsh and GSK-3β.

I began by using the western assay protocol previously described in section 4.11 to examine the levels of Dsh. Since phospho-specific antibodies for *Drosophila* Dsh are not available, I looked instead for the presence of a hyperphosphorylation shift in the Dsh band. Using an antibody against Dsh, I did not detect any differences in Dsh protein levels, or an apparent phospho shift, indicating that Dsh is probably not hyperactivated in the absence of Lkb1 [Fig. 5.3].

GSK3β is a negative regulator of the Wg pathway, and GSK3β phosphorylated on serine 9 is an inactivated version of GSK3β (Cross et al., 1995). Active GSK3β has a role in the ubiquitin mediated degradation of Arm (Aberle et al., 1997), and since the stabilisation of Arm is a post-transcriptional mechanism, this mechanism may be inhibited in *lkb1* clones, thus leading to an accumulation of Arm. Examination of total GSK3β protein levels and the activation status of GSK3β using a serine-9 phospho-GSK3β specific antibody revealed that these were unchanged, further indicating that the Wg pathway is not hyperactivated in *lkb1* clones [Fig. 5.3].

Although previous data from *Xenopus* (Ossipova et al., 2003) and mammalian cell lines (Spicer et al., 2003) have demonstrated a role for Lkb1 in the regulation of the Wnt signalling pathway, I was unable to find evidence of excess Wg signalling in pupal retinas deficient for Lkb1, suggesting either that Lkb1 mediated regulation of Wnt signalling demonstrated in *Xenopus* and mammalian cell lines is not conserved in *Drosophila*, or that this interaction is not conserved in this eye specific context.

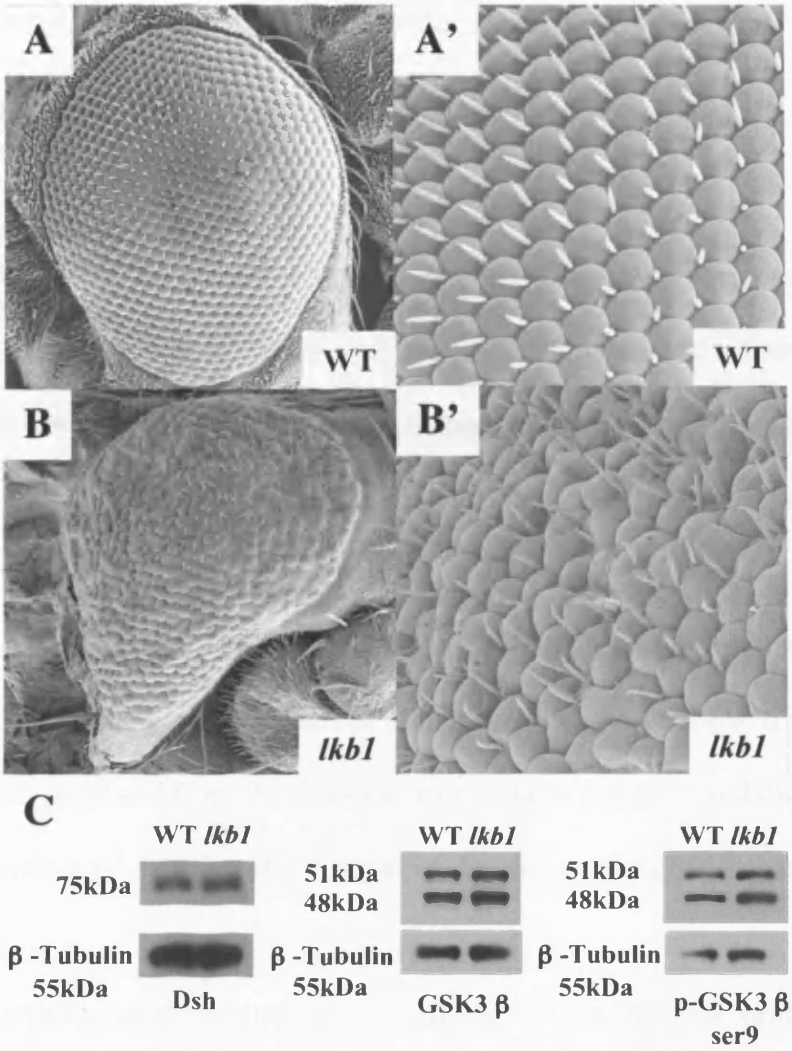



**Fig. 5-3 The Wg pathway is not activated in *lkb1* clones**
(A, A') SEMs of a wildtype eye show show a regular array of ommatidia and bristles. (B, B') SEMs of the *lkb1 4A4-2* eye. The eye is wholly mutant for *lkb1*. Defects include fused ommatidia, missing and excess bristles, and disorganised bristles. The eye is also smaller, rougher, and misshapen. Loss of bristles in the retina can indicate an upregulation of Wg signalling, however, bristles are still present in *lkb1* mutant eyes. (C) Western analysis of Wg signalling pathway components suggest that this pathway is not activated in *lkb1* clones. Total levels of Dsh, and GSK-3β, and the activation status of GSK-3β as measured by phosphorylation on its serine-9 motif, were also unchanged in *lkb1* retinas. Tubulin is the loading control.

### 5.2.2 Stabilisation of Armadillo does not occur through an increase in DE-cadherin protein levels

E-cadherin has been shown to have a stabilising effect on β-catenin in AJs, and also has a role in recruiting β-catenin to the AJs (Gottardi et al., 2001; Kuphal and Behrens, 2006). Therefore, I reasoned that an increase in DE-cadherin (*Drosophila* E-cadherin homologue) levels might be a possible mechanism for the stabilisation of Arm in *lkb1* clones.

I examined the distribution of DE-cad in 40% pd retinas containing *lkb1* clones. Co-staining these retinas with DE-cad and Arm showed that DE-cad was mislocalised from the junctions in a fashion that mirrored the mislocalisation of Arm, but did not seem to be independent of Arm staining [Fig. 5.4].

Further analysis by immunoblotting showed that the total protein levels of DE-cad was unaltered in *lkb1* retinas. I next examined the protein levels of D-cat (the *Drosophila* α-catenin homologue), and similarly found no change in the abundance of this protein.

Since the levels of DE-cad, and D-cat are unchanged, I concluded that it is unlikely that any of these junctional proteins are stabilising factors that could result in the increase in Arm. Similarly, Baz, also a junctional protein, did not show an increase in abundance [Fig. 4.17], suggesting that stabilisation of Arm at the junctions may be an unlikely mechanism for the observed

increase in Arm. However, additional junctional components could be examined in this way to further test this possibility.

Having determined that the increase in Arm was not transcriptional, I had tried to identify a post-transcriptional mechanism for the stabilisation of Arm. I looked at Wg signalling and compared the relative abundance of junctional components, but I was unable to identify a possible mechanism for the stabilisation of Arm.

Other mechanisms left unexplored are the degradation of Arm by other means, e.g. an increase in phosphorylation by CKI, and possibly direct regulation of Arm degradation by Lkb1. β-catenin has a number of phospho motifs which are known to be involved in its ubiquitin mediated degradation. The Ser45 motif, modified by CKI, primes the protein for further phosphorylation by GSK3β on the sites Ser33, Ser37, Thr41 (Amit et al., 2002). Additionally, Lin-Marq *et al,* showed that the expression of wildtype LKB1 could result in an increase in β-catenin phosphorylation by GSK3β (Lin-Marq et al., 2005).

Since these sites are conserved in *Drosophila* Arm, I obtained a number of antibodies to examine these sites in *lkb1* mutant retinas, to see if I could identify an alteration in phosphorylation at these motifs. Unfortunately, despite repeated attempts with these antibodies, I could not obtain a clear enough signal to assess the phosphorylation status of these sites.

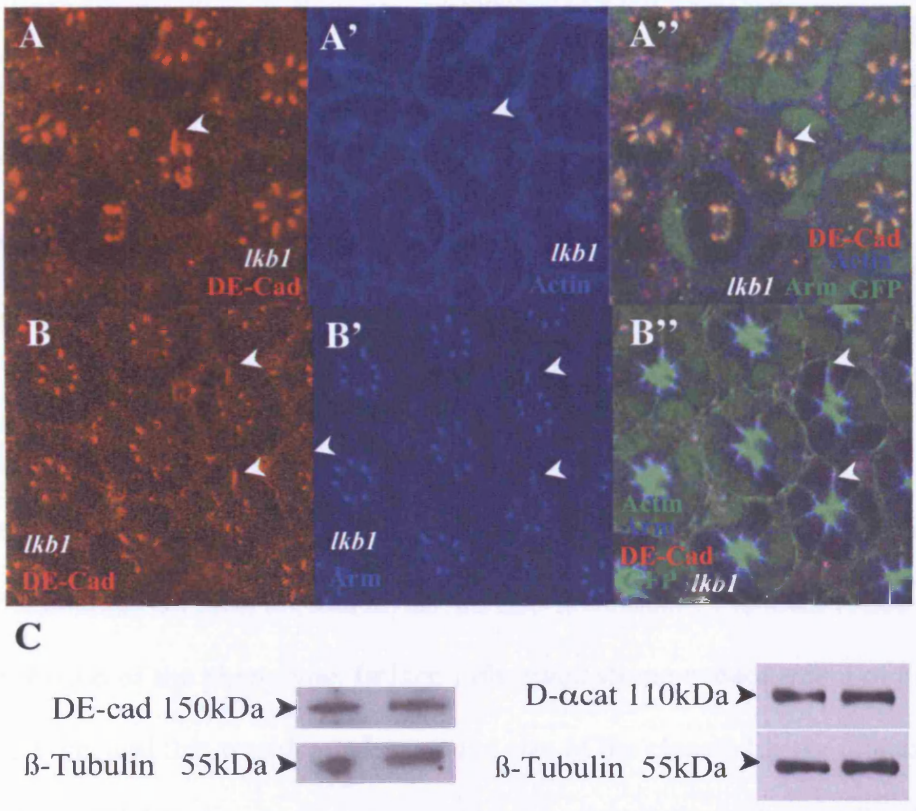



**Fig. 5-4 DE-cadherin is mislocalised but not stabilised in *lkb1* clones.** DE-cadherin (red) was mislocalised in 30% (A) and 40% pd (B) *lkb1* clones (white arrowheads). These defects consistently coincide with Arm mislocalisation. (C) DE-cadherin, and Drosophila alpha-catenin (D-αcat) do not show a difference in protein abundance in *lkb1* mutant retinas. Tubulin is a loading control.

## 5.3 Lkb1 genetically interacts with Armadillo

Previous work by Ahmed *et al* demonstrated that excess Arm in the retina could lead to missing PRCs, and misshapen rhabdomeres, similar to that seen in *lkb1* clones (Ahmed et al., 1998). I wondered if part of the *lkb1* phenotype could be attributed to the excess of Arm observed by immuno-blotting.

In order to test this, I reduced the gene dosage of *arm* by 50% using the null allele *arm4*, in *lkb1* clones in the retina. Since a consistent feature of the *lkb1* phenotype is the mislocalisation of Arm, and biochemical analysis has demonstrated that Arm protein levels are also increased, I expected to see a suppression of the phenotype. In fact, I observed strong enhancement of the phenotype, and this was dependent on the size of the clones. Larger clones in particular exhibited a more severe phenotype, particularly in the middle of the clones. There also seems to be a more penetrant loss of PRCs than is observed in *lkb1* clones alone, although this was difficult to determine since the morphology of the PRCs were so severely disrupted. Ommatidial patterning was completely lost within these clones, and in addition rhabdomeres, where visible, were misshapen [Fig. 5.5]. This result was unexpected since my initial experiments demonstrated that Arm protein levels are increased in the absence of Lkb1.

The data presented here suggest that Lkb1 and Arm may function in similar pathways or processes in the eye to regulate polarity. However, I could not reconcile the data presented here with that observed in my immuno-

histochemical and biochemical assays of Arm, which suggest that decreasing the levels of Arm in *lkb1* clones should lead to a suppression of the phenotype, however, in light of recent efforts to reproduce this result, this result may be more consistent with a decrease of Arm localisation at the junctions.

Further analysis of the biochemical basis of the interaction between Lkb1 and Arm is necessary to explain the results of this assay.

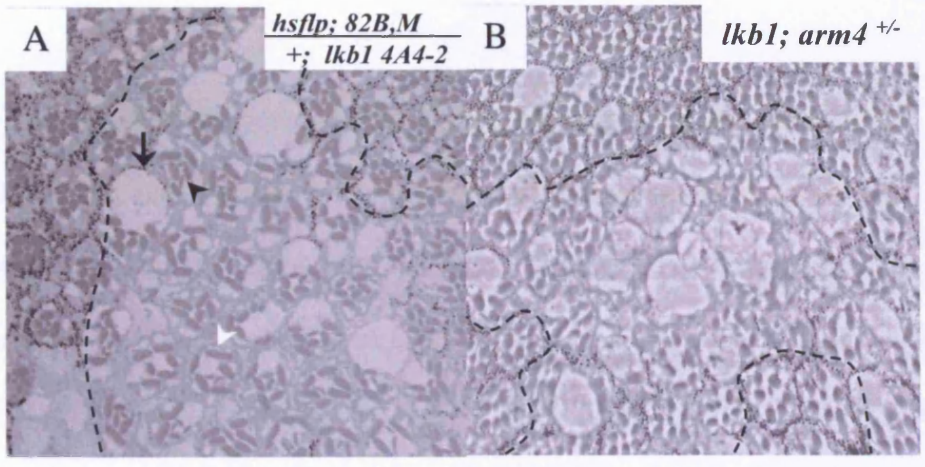



**Fig. 5-5 Arm genetically interacts with Lkb1.** Clones are marked by a lack of pigment granules and are contained within the dashed black lines. (A) The *lkb1* phenotype in the eye, includes enlarged cell bodies (black arrow), missing PRCs (black arrowhead), and misshapen rhabdomeres (white arrowhead). (B) Clones of *lkb1* haplo-insufficient for *arm,* using the null *arm 4* allele, exhibited a dramatic increase in morphogenetic defects, and rhabdomeres and ommatidia were no longer visible in the middle of clones.

## 5.4 AJs in *lkb1* pupal retinas are often longer, more numerous and show ectopic localisation

Immuno-flourescence data suggests that Arm, Baz, and DE-cad are mislocalised, and in particular, frequently show an expansion of staining towards the basal domain. Given that Arm, Baz and DE-cad are major components of the AJs, and have established roles in the biogenesis/assembly of AJs (Gottardi et al., 2001; Harris and Peifer, 2004; Harris and Peifer, 2005; Kuphal and Behrens, 2006), I reasoned that an expansion of Arm at the lateral membrane may lead to an attendant expansion of the AJs in *lkb1* PRCs.

In order to determine whether adherens junctions were also expanded, or whether there were any other defects in the AJs at these stages, I looked at ultrathin (70nm) sections of *lkb1* pupal retinas at 50% pd. At 50% pd, the pigment that differentiates between wildtype and mutant retinas becomes apparent, and therefore is the earliest stage at which I can study these retinas.

By this stage many defects are apparent; with respect to the AJs, 3 major features of this phenotype are evident 1. *lkb1* PRCs often have longer AJ than that seen in wildtype PRCs 2. All wild type cells have 2 junctions each, but in *lkb1* clones, more than two AJs per cell can frequently be observed. Some AJs are clearly separate from each other, e.g. where AJs appear at four corners of a single cell; in other cases many smaller junctions appear next to each other, these may be a product of a single AJ which has fragmented 3. AJs in wild type pupal retinas uniformly occupy an apico-lateral position. In *lkb1* retinas, as

well as exhibiting lateral expansion, ectopic junctions also occasionally appear on the apparent lateral and basal membranes [Fig. 5.6].

In order to determine if the apparent increase in AJ length in *lkb1* PRCs was real, I measured a number of AJs both in wildtype PRCs (n=21) and in *lkb1* PRCs (n=31), using the Adobe Photoshop measure tool. Histogram presentation of the data showed that while the average length of the wildtype AJ was 0.55μm, *lkb1* AJs were over double the length at 1.28μm, and in addition, *lkb1* junctions also displayed a wider variation in length.

To examine the distribution of *lkb1* AJ length more closely, I displayed the data as a box plot graph. Box plot analysis reveals that the majority of mutant junctions (approximately 75%) are notably longer - on average junctions are 2.31 times longer, with a small number of outliers that display longer and shorter junctions. The shorter junctions are possible a result of junction breakdown, often displaying as a string of 'mini' junctions along the apico-lateral membrane [Fig. 5.6].

This data suggests that Lkb1 is required for the proper localisation of AJs at the apico-lateral membrane, for the integrity of AJs - since the loss of *lkb1* can also lead to the appearance of fragmented AJs, and for the restriction of AJs to their appropriate length.

An alternative explanation for these defects is that defects in the conformation and structure of PRCs may lead to an apparent increase in AJ length or mislocalisation. To test this theory I attempted to examine the proximo-distal structure of 50% pd *lkb1* ommatidia, however technical difficulties precluded further examination of lateral PRC structure in TEM sections. The pupal retina is a more flexible tissue than the adult retina, and

despite numerous attempts, obtaining lateral sections was very difficult, and unfortunately most attempts resulted in oblique sections that did not fully display the full length of the PRC.

Since the levels of DE-cad, and D-cat are unchanged, it is unlikely that either of these proteins drive the formation of expanding junctions. The apparent increase in Arm at the junctions in immuno-fluorescence images, taken together with this data from pupal TEMs is intriguing, since it may suggest that stabilised Arm in *lkb1* retinas preferentially incorporate into junctions.

This brings about the next question: is an increase in Arm protein levels sufficient to precipitate the expansion of AJs in *lkb1* clones, or is this expansion due to some other, rate limiting factor for the formation of AJs, such as an increase in other components of the AJs. Since I did not find a change in the levels of other junctional components, I next wanted to see if an increase in Arm alone could indeed lead to expanded junctions in PRCs.

In order to determine this, and to assess the effects of over-expressed Arm on AJ morphology I attempted to overexpress full length Arm in PRCs using the flip out technique. I crossed *hsFlp; UAS-Arm.WT* flies to a flip-out stock of the genotype *yw; Act5C<FRT y FRT>Gal4, UAS GFP.* The offspring of this cross constitutively express GFP and Arm in clones. I examined larvae from this cross for the presence of GFP, a marker in this system for gain of function clones, however, despite repeated attempts to induce *UAS-ArmWT* gain of function clones, and using different hsFlp stocks, I did not find larvae expressing the *UAS-GFP* and *UAS-ArmWT* transgenes. I believe this may have

been due to ectopic cell death of the gain of function clones, induced by the overexpression of Arm (Ahmed et al., 1998).

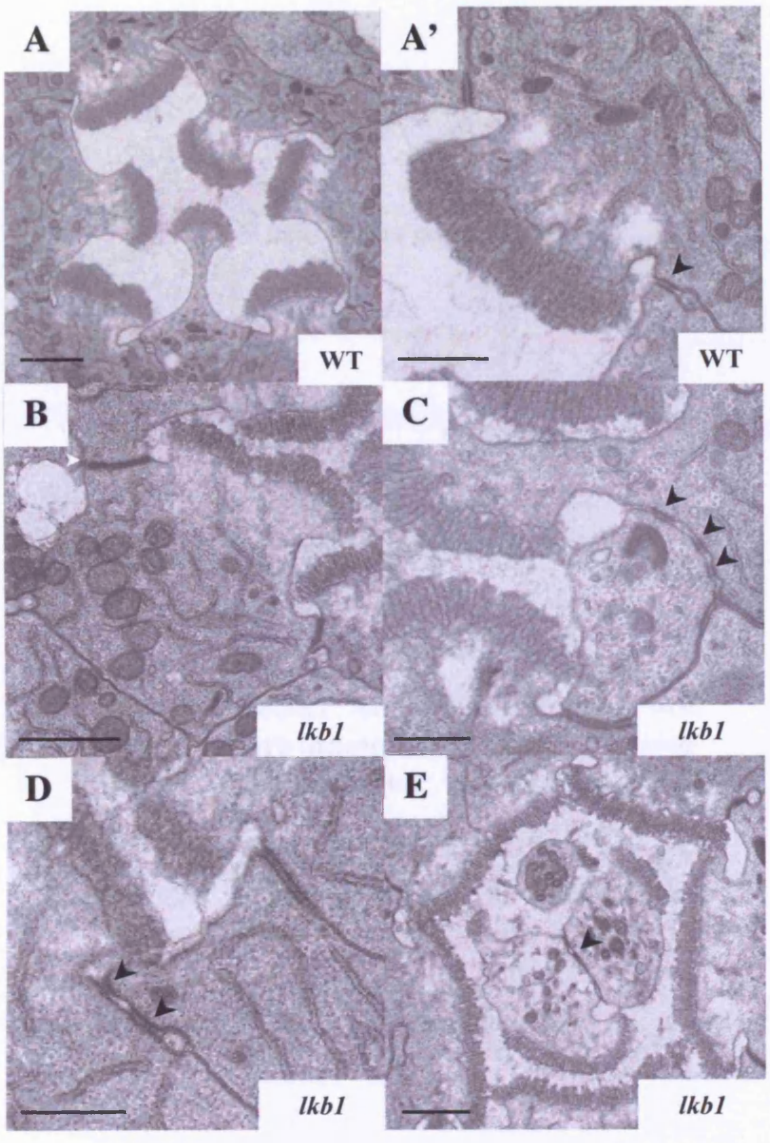



**Fig. 5-6 Adherens junctions are longer, more numerous and mislocalised in *lkb1* photoreceptor cells.** (A, A') Ultrathin sections (70nm) of a wildtype ommatidium at 50% pd. Adherens junctions (AJs) in wildtype PRCs occupy an apico-lateral position in the cell, and each cell has 2 AJs (black arrowhead), of uniform length (0.5μm). (B-E) AJs in *lkb1* PRCs are frequently longer (white arrowhead in B), are sometimes disjointed (black arrowheads in C and D), and are often present in greater numbers in the cell. AJs are also occasionally mispositioned from an apicolateral to lateral or basal position (black arrowhead in E). Scale bars represent 1μm in A,B,E; and 0.5μm in A',C,D.

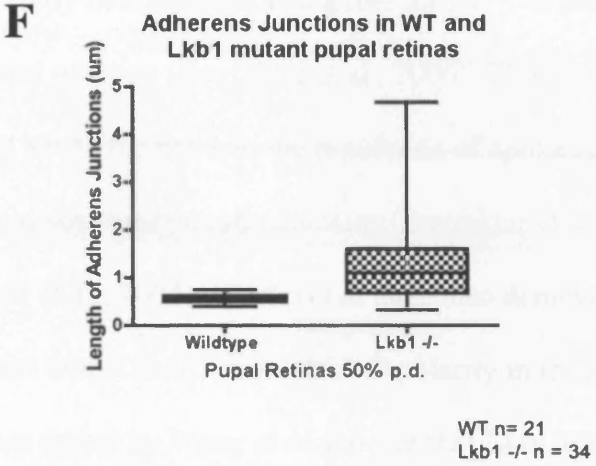



**Fig. 5-6 (cont) Adherens junctions are longer, more numerous and mislocalised in *lkb1* photoreceptor cells.** (F) Boxplot analysis of *lkb1* AJ length in PRCs in 50% pd pupal retinas. The average length of AJs in *lkb1* clones is in general increased to 1.28μm, however AJs also exhibit an increased range in length. Smaller junction lengths may indicate fragmented AJs.

## 5.5 Par-1

A recent study has demonstrated a role for Par-1 in the restriction of AJs to an apico-lateral position (Bayraktar et al., 2006). This and additional studies further revealed a role for Par-1 in the regulation of epithelial polarity and the development of distinct membrane domains (Bayraktar et al., 2006; Bohm et al., 1997; Suzuki et al., 2004). Martin *et al* have also demonstrated that Par-1 can act in concert with Lkb1 to regulate A-P polarity in the *Drosophila* oocyte, and a very recent report by Wang *et al* showed that Lkb1 is required for the activation of Par-1 in *Drosophila* (Wang et al., 2007a).

Since the *par-1* phenotype demonstrates a subset of the *lkb1* phenotype, and reports have shown that Par-1 can be regulated by Lkb1 both in invertebrate and vertebrate studies, Par-1 thus represents a potential candidate protein by which Lkb1 might be acting to regulate the localisation of AJs in PRCs, and the development of epithelial polarity.

### 5.5.1 *Par-1* loss of function clones show defects in PRC morphology and Armadillo localisation

Since *par-1* and *lkb1* loss of function clones show similar defects in the oocyte, I wondered if *par-1* loss of function clones in the eye would phenocopy *lkb1* clones. A colleague in the lab created loss of function Par-1 clones in the pupal eye using the excision null allele *w3*. Clones in 40% pd retinas showed

defects in Arm localisation and actin organisation that reproduced to a lesser degree the defects in Arm and actin seen in *lkb1* clones. Arm showed expansion towards the basolateral membrane, and PRCs, although not as severely affected as *lkb1* mutant PRCs, also occasionally showed abnormal morphology [Fig. 5.7].

Further phenotypic similarities to the *lkb1* phenotype were evident in *par-1 w3* adult clones generated in the retina. *par-1* clones showed morphogenetic defects in rhabdomere, and loss of PRCs, and in addition also frequently displayed large cell bodies, similar to those observed in *lkb1* clones [Fig. 5.7].

These results suggest that Par-1 may have a role in regulating polarity and morphogenetic processes in the eye, and that Lkb1 may regulate Arm localisation through Par-1. In addition, these results are also consistent with a role for Par-1 in regulating morphogenetic processes in the retina, suggesting that Par-1 and Lkb1 may also cooperate in these processes in the retinal epithelium.

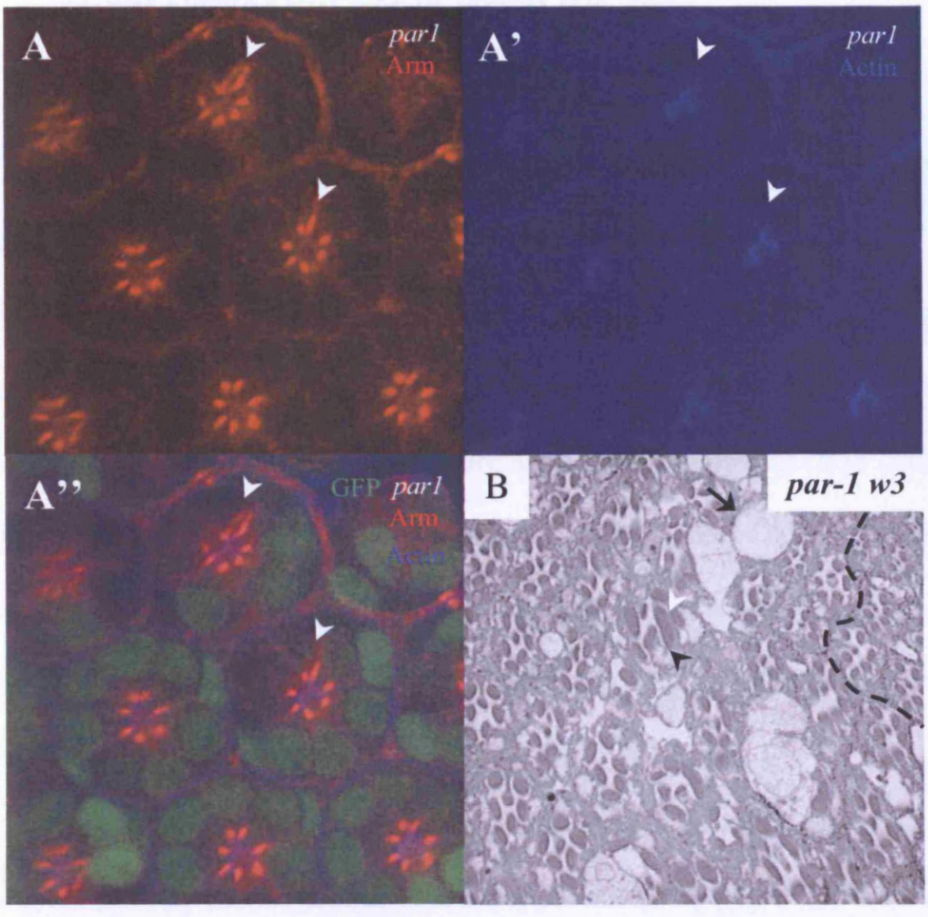



**Fig. 5.7 *par1* clones show defects in Arm distribution in 40% pd pupal retinas.** (A-A'') GFP marks wildtype tissue. Arm (red) shows basal expansion in *par1 w3* clones (white arrowhead) (experiment conducted by Nancy Amin). (B) *par-1 w3* clones are identified by the lack of pigment cells and are to the left of the black dashed lines, *par-1 w3* clones exhibit defects in PRC morphology (black arrow), misshapen rhabdomeres (white arrowhead) and missing PRCs (black arrowhead).

### 5.5.2 Par-1 is stabilised at the basolateral membranes in *lkb1* pupal retinas but not in larval eye discs

Since Par-1 and Lkb1 show similar loss of function phenotypes in the eye, it is possible that a function of Lkb1 may be to regulate Par-1.

Using an antibody raised against a C terminal fragment of hPar-1b, I began my analysis of Par-1 in *lkb1* clones by examining Par-1 distribution in *lkb1* clones in the larval disc. Since there are no observable defects in cellular morphology, or in the distribution of polarity markers Arm, DaPKC and Baz in the larval disc, I did not expect to find alterations in Par-1 abundance or distribution. Accordingly, there was no observable change in Par-1 protein in *lkb1* clones in the larval eye disc [Fig. 5.8].

Since previous data has indicated that defects in morphology and epithelial polarity in *lkb1* clones become apparent during pupal development rather than in the larval stages, I next looked at Par-1 distribution in early pupal retinas. I observed a consistent upregulation of Par-1 in *lkb1* clones in 30% pd retinas [Fig. 5.8]. Closer inspection revealed that Par-1 shows uniform increased abundance at the basal membrane and also increased localisation at the lateral membranes, which, as highlighted by Par-1, appeared to be more disorganised in *lkb1* mutant PRCs. 40% pd retinas containing *lkb1* clones also reveal continued upregulation of Par-1 in *lkb1* clones, and this upregulation could be observed mainly at the basal membrane.

Thus immuno-histochemical data suggested an increase in the abundance of Par-1 protein in *lkb1* clones. In order to test if Par-1 protein was indeed stabilised in *lkb1* tissue, I next compared total Par-1 protein levels in 50% pd pupal retinas in the previously described western blot assay [Fig. 5.8].

Multiple transcripts are produced from the *par-1* gene due to alternative promoters, multiple 3' ends and other alternative splicing events. The Par-1 antibody used in this assay primarily recognises two Par-1 isoforms in the pupal retina, running at approximately 105 and 115kDa. Both isoforms were upregulated in *lkb1* retinas, showing approximately a two-fold increase. This further served to demonstrate that Par-1 upregulation in early *lkb1* retinas persists through development to at least 50% pd.

Although defects in Arm can be observed as early as 20% pd, as with other defects in *lkb1,* these defects have variable penetrance. Therefore confocal images from 40% pd demonstrate that Arm localisation and cellular morphology can remain unaffected, whilst Par-1 shows increased localisation at the membrane. This suggests that the observed increase in Par-1 may be epistatic to defects in Arm localisation or cellular morphology, and also that misregulation of Par-1 in *lkb1* clones may lead to the observed defects in Arm, and morphology.

This data demonstrates that loss of Lkb1 can lead to increased levels of Par-1, which localises at higher levels to the basal and occasionally the lateral membrane.

### 5.5.3 In the absence of *lkb1*, phosphorylation of Par-1 at an inhibitory site is reduced

Studies have shown that the localisation of Par-1 at the membrane can be regulated by phosphorylation events. One such modification is mediated by DaPKC; this site, when phosphorylated, prevents the activation and tethering of Par-1 at the membrane (Hurov et al., 2004; Suzuki et al., 2004). This site, T595 in mammalian Par-1b and T564 in Par-1a, despite being located in a divergent region, is conserved in *C. elegans* and *Drosophila* Par-1, demonstrating its importance in Par-1 function (Suzuki et al., 2004; Vaccari et al., 2005).

Probing a western blot of wildtype and *lkb1* mutant retinas with an antibody against this site revealed that although absolute amounts of Par-1 have increased, the relative levels of Par-1 protein phosphorylated at this site has decreased [Fig. 5.8]. A decrease in this phosphorylation event would allow more Par-1 to associate with the membrane, and indeed immuno-fluorescence data confirms that while Par-1 is normally restricted mainly to the basal membrane, in *lkb1* mutant clones there appears to be excess Par-1, both at the lateral and basal membranes.

This data puts forward an interesting model for the increased levels of Par-1 in *lkb1* clones. These results suggest that the increased stability of Par-1 protein in *lkb1* mutant retinas may in fact reflect increased association of Par-1 at the membrane, since association with the membrane may protect Par-1 protein from normal routes of degradation. Although this site also has a

reported function in inhibiting Par-1 activity, further work is required to establish the activity status of Par-1 in *lkb1* clones.

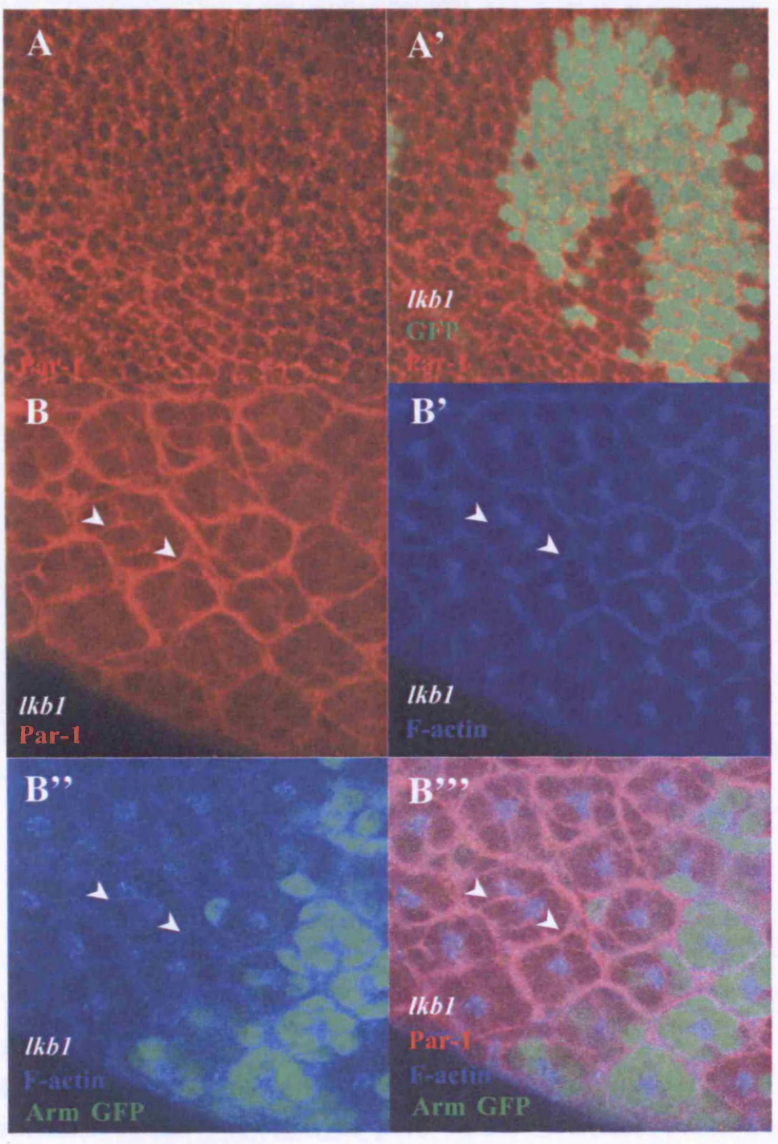



**Fig. 5-8 Par-1 is stabilised and dephosphorylated in *lkb1* retinas.** (A, A') Par-1 did not show an increase in protein levels in *lkb1* clones in the third instar eye disc. However, at 30% (B) and 40% pd (C), an increase in Par-1 levels is apparent in *lkb1* clones. Par-1 is upregulated primarily at the basolateral cortex (white arrowheads).

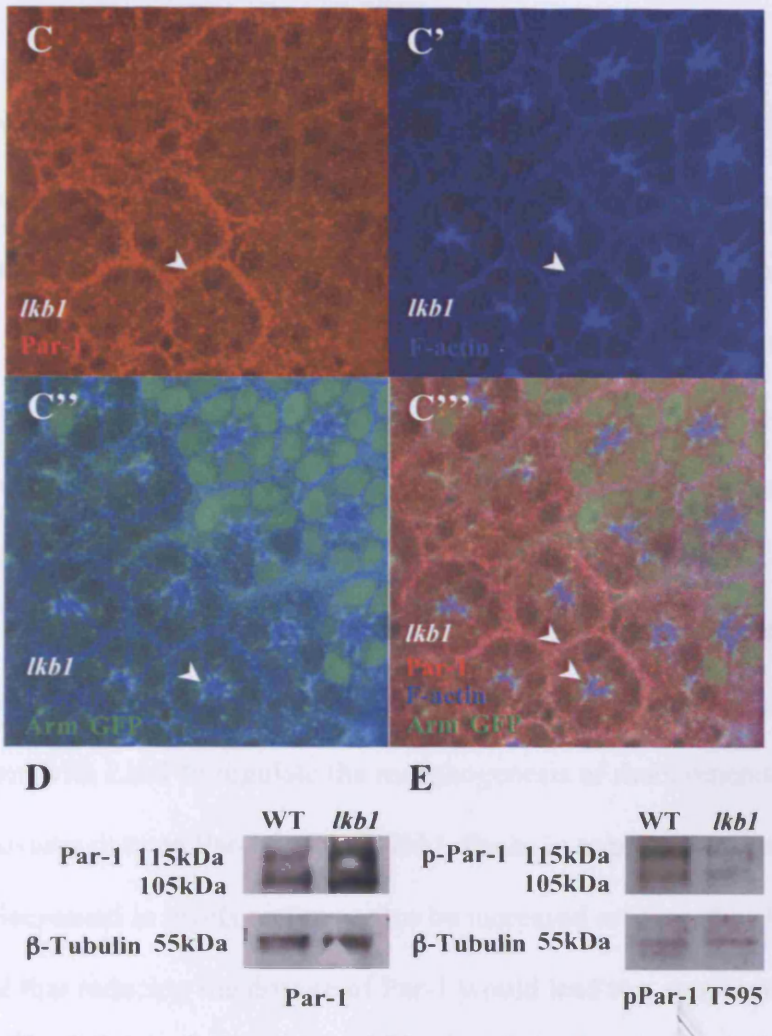



**Fig. 5-8 (cont) Par-1 is stabilised and dephosphorylated in *lkb1* retinas.** However, at 30% (B) and 40% pd (C), an increase in Par-1 levels is apparent in *lkb1* clones. Par-1 is upregulated primarily at the basolateral cortex (white arrowheads). (D) Western analysis of Par-1 levels in *lkb1* retinas indicate increased levels of Par-1 protein. Par-1 isoforms recognised by the antibody are stabilised in *lkb1* retinas. (E) Despite increased levels of Par-1, phosphorylation at a conserved regulatory site known to be phosphorylated by aPKC, is greatly reduced.Tubulin is a loading control.

### 5.5.4 Lkb1 genetically interacts with Par-1

Previous work by Martin *et al* has suggested that Par-1 interacts genetically with Lkb1 in *Drosophila*. In order to test this interaction in the eye, and to test the immuno-histochemical and biochemical data I had generated, I reduced the gene dosage of Par-1 by 50% in clones in the adult eye which were deficient for *lkb1* using the *par-1* null allele *w3* [Fig. 5.9]. Since Par-1 protein levels are increased in *lkb1* clones, I expected to see a suppression and partial rescue of the phenotype: interestingly, lowering the dosage of *par-1* instead led to an enhancement of the *lkb1* phenotype. The morphogenetic defects seen in *lkb1-/-*; *par-1 +/-* rhabdomeres were more severe, indicating that Par-1 cooperates with Lkb1 to regulate the morphogenesis of rhabdomeres.

Previous data on Par-1 levels in *lkb1* clones in pupal retinas suggest that Par-1 is increased in levels and may also be increased activity, thus I had predicted that reducing the dosage of Par-1 would lead to a suppression of the phenotype, therefore this was an unexpected result.

However, this result may have been obtained for the following reasons: firstly, since this genetic interaction is based on null alleles, a complete loss of both proteins may result in synthetic defects that do not accurately represent potentially complex physiological interactions between Lkb1 and Par-1. Although Par-1 has increased in abundance, the activation status of Par-1 is unclear, and certainly an increase in levels does not reflect an increase in Par-1 activity. Thus, this data may also imply that although the inhibitory phosphorylation of Par-1 appears to be reduced, Par-1 is inactivated in *lkb1*

clones and thus reducing Par-1 would be expected result in an enhancement of the phenotype.

Secondly, since the loss of *par-1* in pupal and adult clones show phenotypic similarities to that observed in *lkb1* clones, it is likely that a reduction in *par-1* in a background deficient for *lkb1* would result in a synergistic enhancement of the phenotype.

However, the results of genetic interaction experiment are consistent with results obtained from immuno-fluorescence studies of Par-1 in *lkb1* clones, which suggest that Par-1 is downstream of Lkb1, and that Par-1 and Lkb1 cooperate to regulate epithelial polarity and morphogenesis in the eye.

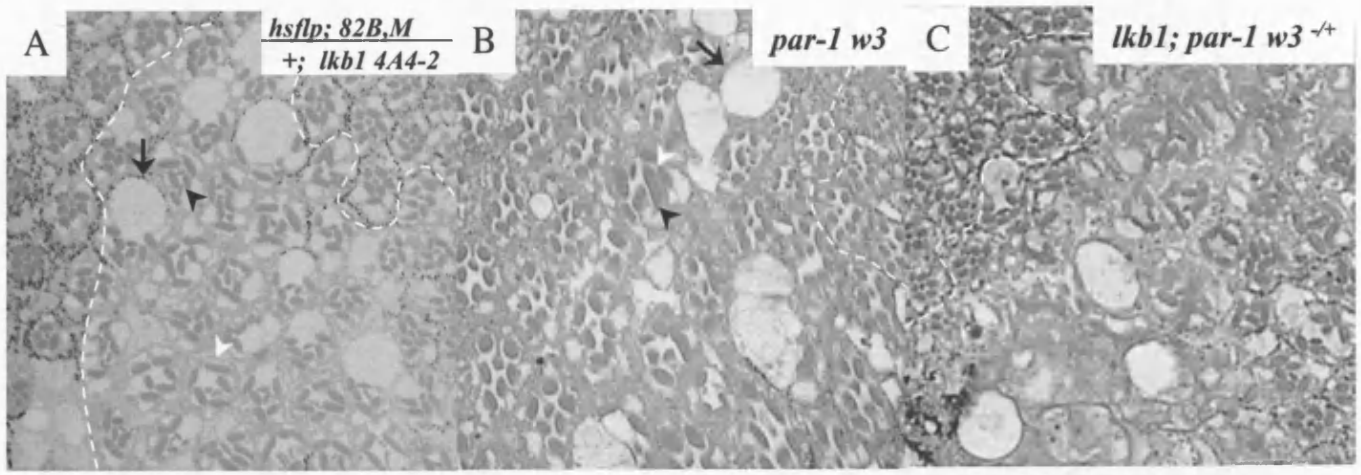



**Fig. 5-9 *par-1* genetically interacts with *lkb1*.** Clones are marked by a lack of pigment cells and are contained within white dashed lines (A) The *lkb1* phenotype in the eye, includes enlarged cell bodies (black arrow), missing PRCs (black arrowhead), and misshapen rhabdomeres (white arrowhead). (B) The *par-1* W3 null phenotype in the eye is similar to the *lkb1* null phenotype, and includes defects in rhabdomeres morphogenesis (black arrowhead), as well as a loss of photoreceptor cells (white arrowhead) and enlarged cell bodies (black arrow) (C) A reduction in the gene dosage of Par-1 using the *par-1 w3* null allele results in an increase in morphogenetic defects in *lkb1 4A4-2* clones.

## 5.6 *lkb1* clones show defects in cone cell configuration, morphology and number

Defects in *lkb1* PRCs are apparent in cross section at 20% pd. In side view images, mutant ommatidia appear to drop out of the plane of the epithelium, suggesting possible defects in attachment to the cone cell roof. Since junctional remodelling and attachments play a large part in cone cell development and positioning, and previous data had suggested defects in AJ formation in *lkb1* mutant clones, I decided to examine cone cell pattern formation and the defects observed in ommatidial attachment to the cone cell roof more closely.

The configuration of cone cells are very precisely regulated by adhesive forces, and governed by mechanisms that minimise surface area (Hayashi and Carthew, 2004). A recent study by Hayashi *et al* showed that the conformation of cone cell groups closely followed that seen in groups of soap bubbles with the same number of separate units, or 'cells'. While soap bubble conformation is determined by surface mechanics, the predefined configuration of cone cell clusters is determined by the differential expression of N- and E-cadherins. The sum result of both mechanisms is to minimise total cell surface area, and the resulting conformation of cone cells in the pupal and adult retina is a cross like arrangement of 4 cone cells, described in section 1.4.2.1 and Fig. 1.6.

### 5.6.1 Ommatidial and cone cell development proceed normally in larval and early pupal *lkb1* clones

I began my examination of cone cell morphology by looking at early stages of development to see if cone cells in *lkb1* mutants were unable to properly complete the movement from below the PRC preclusters to the top of the pre-cluster that occurs during the larval to pupariation transition, a process that involves junctional remodelling. I examined *lkb1* clones in larval eye discs stained for DN-cadherin and Arm. Ommatidial preclusters begin to be specified following the passage of the MF; preclusters at the posterior most region of the disc are the most matured and developed, and in these preclusters the cone cells begin to displace apically to position themselves above the PRC cluster. I examined these ommatidia, marked by DN-cad and Arm, for defects in Arm or DN-cad localisation, or for defects in ommatidial morphology or positioning. I did not find any such defects, and ommatidia at this stage were normally formed, normally positioned at the apical region of the larval disc, and showed normal DN-cad and Arm staining [Fig 5.10].

I next examined clones of *lkb1* in 10% pd retinas. By this stage, cone cells are positioned above the PRCs, and each cone cell cluster displays the classic four-cell configuration, with stereotypical contacts between the four cells [Fig. 1.5 and 1.6]. In *lkb1* clones at this stage, cone cell morphology and arrangement, as highlighted by Arm and DN-cad, appear normal. DN-cad highlights the interface between the cone cells, and also appears normal [Fig. 5.11]. This demonstrates that *lkb1* mutant cone cells are able to shift apically and begin to form a cone cell roof over the developing PRC chamber.

Actin and Arm both localise above the apex of the ommatidia, as well as to the ommatidia. Longitudinal images revealed while Arm was unaffected, actin staining was decreased, and appeared as patchy staining along the top of the epithelium, suggesting that actin organisation is disturbed in *lkb1* clones [Fig. 5.11].

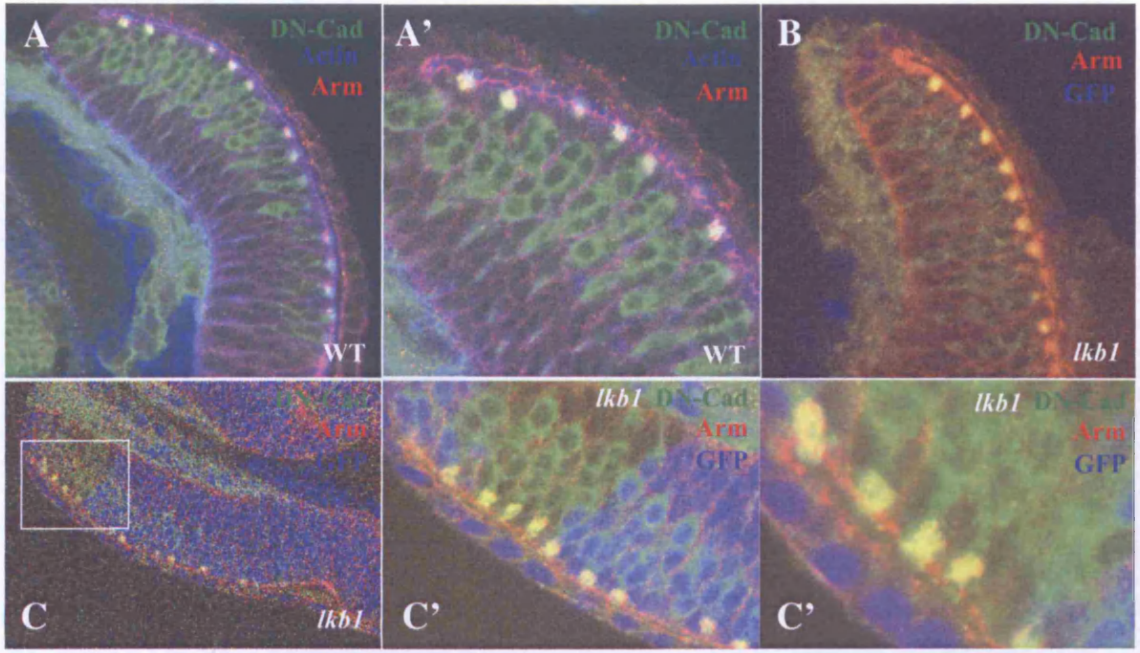



**Fig. 5-10 Ommatidial preclusters form correctly in *lkb1* clones in the larval disc.** GFP (blue) marks wildtype tissue (A,A') Ommatidial preclusters in the wildtype third instar disc. (DN-cad in green, Arm in red, F-Actin in blue) (B-C'') Ommatidial preclusters in *lkb1* clones in the larval disc do not show defects in morphology, or Arm or DN-cadherin expression, and are correctly positioned at the apical surface of the disc. (C',C'') are higher magnification images of the boxed region at the posterior of the disc in C. (DN-cad in green, Arm in red, GFP in blue).

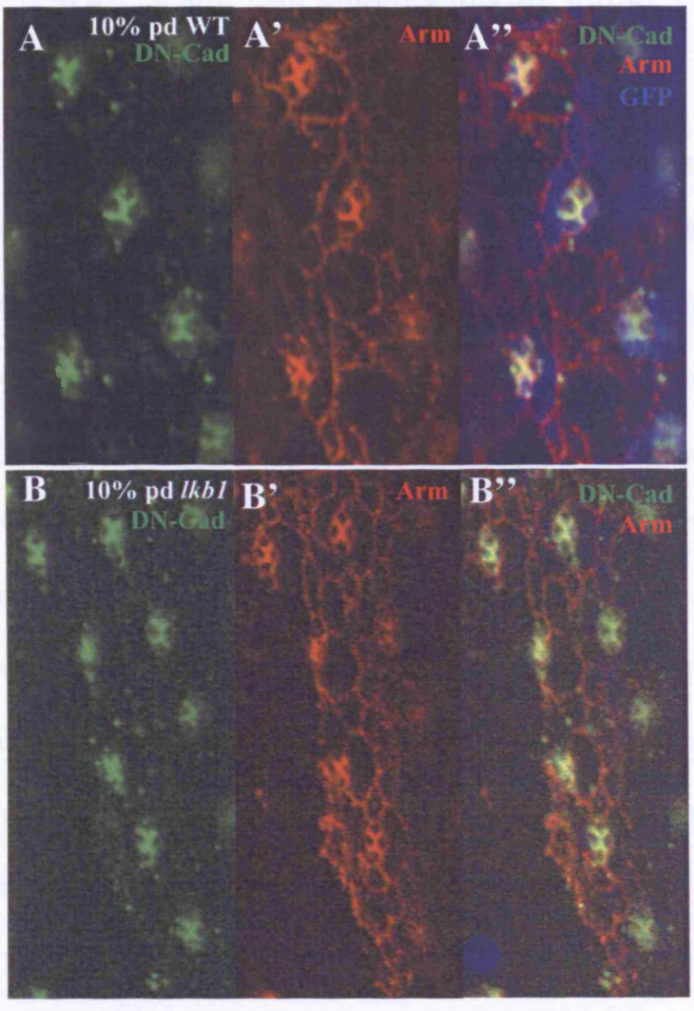



**Fig. 5-11 Cone cells form correctly in early *lkb1* pupal clones.** GFP (blue) marks wildtype tissue (A) Wildtype cone cell groups have a typical configuration of 4 cone cells arranged in a cross pattern. (B) Cone cell groups form correctly at 10% pd in *lkb1* clones, and with the correct number of cone cells per group.

### 5.6.2 Lkb1 is required for the maintenance of cone cell configuration and morphology during pupal development

At approximately 17% pd, clones deficient for Lkb1 show striking defects in cone cell formation; cone cells are frequently missing from the normal group of four, indicating ectopic cell death, since cone cells initially form normally. Excess cone cells were also sometimes apparent, and occasionally a fusion of two or more cone cell groups could be seen. I also observed defects in cone cell morphology, with cone cells appearing rounded and DN-cad often appearing as a closed circle of expression instead of the normal semi-circular expression pattern [Fig. 5.12]. Arm staining did not reveal any defects in Arm localisation or levels; despite the severe defects in *lkb1* cone cell morphology, the localisation and levels of DN-cadherin and Arm were mostly normal, although DN-cad occasionally showed expression throughout the cortex of rounded cone cells.

*lkb1* cone cells at 20% continues to exhibit severe defects in morphology and configuration. Again I frequently observed a loss of cone cells from the normal cluster of four. Excess cone cells, e.g. five cone cells per cluster were also often seen, as well as fusion between cone cell clusters [Fig. 5.12]. I could also detect an alteration in actin staining in *lkb1* clones; as in 10% pd clones, actin appeared to be greatly decreased at the apex of the mutant ommatidia, while uniformly localised in wildtype tissue. Again, Arm expression patterns appeared normal with no apparent defects in Arm in side

view, although cross section images revealed basal mislocalisation as described previously [Fig. 5-1].

This preliminary examination of cone cell defects in *lkb1* mutants demonstrate that Lkb1 has a necessary role in the regulation of cone cell development, and that defects in actin organisation may contribute to defects in cone cell morphology; whether Lkb1 function in cone cell development is also mediated through the control of junction formation and remodelling, or a more specific regulation of cone cell pattern determinants such as Roughest, or Hibris, remains to be determined (Bao and Cagan, 2005)

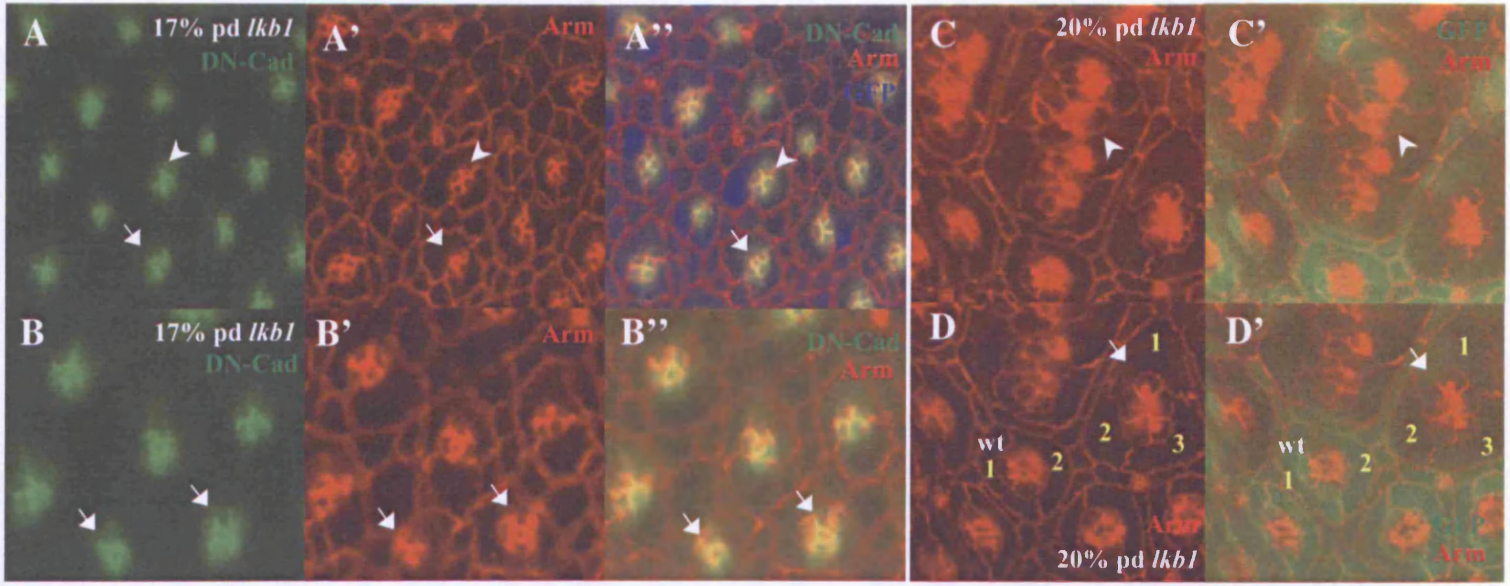



**Fig. 5-12 Cone cells mutant for *lkb1* show defects in configuration and number after early pupal development.** (A) At 17% pd, defects in cone cell formation become apparent. Mutant cone cell groups (white arrow) lacked the archetypal arrangement of 4 cone cells that is observed in wildtype cone cells (white arrowhead in A-A''). Cone cells frequently appeared rounded, and cone cell groups were sometimes missing a variable number of cone cells (white arrowheads in B-B''). (C-D') At 20% pd, in addition to defects in cone cell arrangement, cone cells groups were also occasionally fused (white arrowhead in C and C'). Excess cone cells (white arrow in D and D') and primary pigment cells (yellow numbering in D and D') in a single group were also sometimes observed.

## 5.7 Discussion and conclusions

In this chapter I have presented data that suggest roles for Lkb1 in the regulation of Arm and Par-1, and in the correct formation of AJs and cone cell patterning.

*Arm is mislocalised and stabilised in the absence of lkb1*

I demonstrated that the loss of Lkb1 can lead to severe defects in Arm localisation; defects in Arm can first be observed in *lkb1* clones in 20% pd retinas, and persist throughout pupal development. In addition, the mislocalisation of Arm accompanies every observed instance of abnormal PRC morphology, suggesting that Arm mislocalisation may partially lead to the morphogenetic defects seen in *lkb1* clones. Since immuno-fluorescence revealed an apparent increase of Arm protein at the AJs, I wanted to see if Arm protein was also increased in *lkb1* retinas. I found that Arm was stabilised, and RT-PCR analysis by a colleague in the lab revealed that this observed stabilisation is post-transcriptional. Since these experiments, attempts have been made by a colleague in the lab to reproduce the western blot results that show a stabilisation of Arm. Thus far, experiments have been inconclusive, and we currently await further data.

However, my results indicated that Arm was stabilised in the absence of *lkb1*, and I had repeated these experiments numerous times, and obtained consistent results. Thus, I next attempted to uncover the mechanism by which

Arm is stabilised in *lkb1* retinas. Arm can be stabilised by a number of mechanisms, and I proceeded to examine three.

One of the primary methods by which Arm is stabilised is by the activation of Wg signalling. Activated Wg signalling leads to an inhibition of GSK3β, which is involved in the degradation of Arm protein. I looked at the activation status of GSK3β, and could not detect a change in either total protein levels, or in the inhibitory phosphorylation of GSK3β, suggesting that Arm stabilisation is not occurring through an inhibition of normal GSK3β function. I also looked at Dsh protein levels, and examined the Dsh band for a hyperphosphorylation shift as evidence that Wg signalling may be upregulated in *lkb1* clones, but again, saw no change.

A putative role for Lkb1 in Wg signalling has also been attractive to many researchers since deregulated Wg signalling has been implicated in many colorectal malignancies. Although I could not identify a change in Wg signalling in this system, other studies in mammalian systems and *Xenopus* have reported alteration in Wnt signalling (Lin-Marq et al., 2005; Ossipova et al., 2003; Spicer et al., 2003). Since many other interactions have been shown to be conserved between mammalian and *Drosophila* Lkb1, it is possible that Lkb1 regulates Wg signalling in *Drosophila*. Other assays to study this further that I did not employ include examining the abundance of Wg signalling targets, and further examination of this may yet reveal an alteration in Wg signalling that was not detected in my assays.

A second method by which Arm may be stabilised is by the loss of Lkb1 regulated degradation of Arm. Lkb1 may itself earmark Arm for degradation by phosphorylating the residues on Arm that target it for

ubiquitination and degradation by the proteosome. Since these motifs are conserved in Arm, I attempted to compare levels of phosphorylation in mutant retinas versus wildtype retinas. Unfortunately, technical difficulties with the antibody as discussed above, prevented further examination of this model; therefore I was unable to determine if the degradation of Arm was impaired in *lkb1* clones by this mechanism. An alternative method to examine this may be to look at the ubiquitin levels of Arm in cells expressing Lkb1, however this may also be technically difficult to assess, since beta-catenin ubiquitination occurs at relatively low levels (Aberle et al., 1997; Kitagawa et al., 1999).

The third mechanism I examined was that of stabilisation of Arm at the junctions by other junctional components. β-catenin can be stabilised at the AJs by E-cad (Gottardi et al., 2001; Kuphal and Behrens, 2006). I examined the total levels of DE-cad and D-cat and found that these junctional components were not altered in abundance in *lkb1* retinas, suggesting that stabilisation of Arm by these components was unlikely. Thus I was unable to determine a mechanism for the stabilisation of Arm.

What are effects of stabilised Arm? Previous reports have demonstrated that the overexpression of Arm can lead to defects in rhabdomere morphogenesis and ectopic cell death (Ahmed et al., 1998), hence I wanted to test whether excess Arm in *lkb1* could be responsible for some aspects of the *lkb1* phenotype. I attempted to overexpress full length Arm using the flip-out method to examine the reported morphogenetic defects seen with overexpressed Arm, and to see if overexpressed Arm would phenocopy aspects of the *lkb1* phenotype. However, I found that I could not produce Arm clones, preventing further analysis of the Arm gain of function phenotype. Since Arm

is reported to induce ectopic cell death, it is probably likely the overexpression of Arm in gain of function clones led to the loss of these clones by apoptosis.

Consequently, I attempted to examine this question using a different method. I next carried out a genetic interaction assay by reducing the amounts of endogenous Arm by 50% in clones null for *lkb1*. In light of previous experiments that had suggested that Arm was increased in *lkb1* clones, I had predicted that if stabilised Arm was contributing to the morphogenetic defects in *lkb1* mutants, decreasing the levels of Arm would lead to a suppression of the phenotype. In fact, the results were not so clear-cut. Morphogenetic defects were strikingly more pronounced.

One explanation that follows may be that although increased in absolute levels, the concentration of functional Arm at the AJs is reduced. Decreasing the levels of Arm may further reduce the amount of Arm that is functional at the AJs. Taking this argument further, this may also explain why larger clones show a more penetrant interaction, since a loss of junctional and thus structural support may manifest itself more severely in larger clones, while smaller clones may be bolstered by supporting wildtype cells with normal junctions.

However, this theory is complicated by the ultrastructural analysis of *lkb1* PRCs which suggest that AJs are increased in length and number in *lkb1* clones. In this case, it may be that a decrease of Arm in *lkb1* clones leads to further defects in the formation of AJs.

Further work is required to clarify the results of this experiment, however, the strong genetic interaction between Arm and Lkb1 indicate that

Arm and Lkb1 may function in similar pathways to regulate the structure and formation of PRCs in the eye.

*Lkb1 has a role in the regulation of AJs in the pupal retina*

In order to test if the expansion of junctional markers along the lateral membrane indicated a loss of junctional components from the AJs, or whether the AJs themselves were physically expanding, I examined the ultrastructure of 50% pd *lkb1* PRCs. Analysis of mutant AJs in *lkb1* pupal retinas exhibit multiple defects: fragmentation, increased length, an excess number of junctions per cell, and strikingly, the ectopic localisation of AJs, which could sometimes be observed at the basal membrane of the cell.

One possible explanation for the apparent expansion of AJ in pupal retina EMs may be conformational defects along the proximo-distal axis of the mutant PRC. Theoretically, cross sections through a contorted or buckled section of a PRC may result in more of the AJ being displaced onto a cross section plane, resulting in an apparent expansion of the junction. However, immuno-fluorescence data of 50% pupal retinas do not appear to show the degree of 'buckling' that would be required to result in the frequently observed expansion of AJs. I presented data in the last chapter that demonstrated that this basal expansion persisted throughout the proximo-distal length of the PRC, and that the expansion in the expression of junctional markers was not likely to be due to severe conformational defects in the PRC along the proximo-distal axis.

Moreover, immuno-fluorescence data examining DE-cad and Baz localisation demonstrate that in addition to Arm, junctional markers expand

basally, lending further support to data that shows an apparent spread of junctions along the lateral membrane.

Since Arm shows defects in localisation from approximately 20% pd, I attempted to examine the ultrastructure of *lkb1* PRCs at this stage to determine whether defects in AJ formation would accompany the earliest detectable instance of Arm mislocalisation. Unfortunately, due to the flexible nature of the young pupal retina, I could not obtain sections where I could be certain ommatidia were in the same plane, thus preventing an accurate assessment of AJ structure.

The AJs are a hallmark of apico-basal polarity, and the loss of AJ integrity in *lkb1,* along with data from the previous chapter, provides further evidence that epithelial polarity is severely disrupted in *lkb1* clones.

AJs in the developing pupal ommatidia are the maturing descendents of AJs first formed in the eye disc as PRCs are recruited. This data therefore does not suggest that Lkb1 has a role in the initial biogenesis of AJs, since PRCs are recruited normally, and cellular polarity and morphology are intact in *lkb1* clones in the eye disc. It is however possible to speculate from the data generated thus far, that Lkb1 may have a role in the appropriate remodelling of the junctions during later pupal development. During retinal morphogenesis, AJs are dynamic structures that, in addition to providing a stabilising force to the PRCs, also act to positively aid cell shape changes (Tepass and Harris, 2007). Therefore, defective junctional remodelling could contribute to the defects seen in cellular morphology in *lkb1* PRCs. Further insight into the mechanisms of Lkb1 regulation of AJ formation will be interesting with regard to the biogenesis of AJs.

A recent study by (Harris and Peifer, 2004) demonstrated that in the primary epithelium of the *Drosophila* embryo, Baz was epistatic to Arm and other apical cues in the establishment of AJs. Since Lkb1 has also been shown to regulate Baz localisation, it is tempting to speculate that Lkb1 may have a role in the initial formation of AJs in the embryo through Baz, as well as Arm. Unfortunately, it is not possible to study the effects of loss of Lkb1 during cellularisation on AJ formation, since *lkb1* embryos arrest before cellularisation (Martin and St Johnston, 2003).

Finally, although junctions serve as one of the earliest cues for directing cell polarity, once established, the activities of additional polarity determinants are required to maintain the integrity of junctions, creating a mutual interdependency of polarity determinants. Therefore the mislocalisation of apical and basolateral determinants may also lead to the expansion of the AJs. Mutants in other polarity determinant proteins also show defects in Arm localisation, although these appear to be restricted to lateral expansion defects; whether these mutants also exhibit an increase in junctional length, number and mistargeting of AJ to the basal membranes has not yet been examined.

*Par-1 is regulated by Lkb1*

Recent reports have demonstrated that the loss of Par-1 can lead to the lateral expansion of AJs in the blastoderm (Bayraktar et al., 2006). Par-1 also has a well-established role in the regulation of epithelial polarity, and Martin *et al* showed that Par-1 and Lkb1 cooperate to specify the A-P axis in the *Drosophila* oocyte. Par-1 therefore represented an excellent candidate through

which Lkb1 may mediate some of its functions in regulating AJs and epithelial polarity.

Having observed defects in both epithelial polarity and in AJ formation, I wondered whether some of these defects could be attributed to the misregulation of Par-1. My results indicate a role for Lkb1 in the regulation of Par-1 localisation and levels in the retina, and I have presented immuno-histochemical, biochemical and genetic data demonstrating this.

Loss of function clones of *par-1* examined in both the pupal retina and in sections in the adult eye demonstrated defects in morphogenesis and in the localisation of Arm that paralleled defects observed in *lkb1* clones, suggesting that Lkb1 and Par-1 may function in similar or the same pathways to regulate PRC morphology. Genetic interaction data provided further evidence that Lkb1 and Par-1 act in concert to regulate polarity in the eye, since morphogenetic defects were enhanced when Par-1 was reduced in *lkb1* clones.

Immuno-fluorescence data demonstrated that Par-1 appeared to accumulate in increased levels at the basolateral membranes in *lkb1* clones. Since recent work has shown that phosphorylation on a conserved DaPKC motif acts to prevent association of Par-1 with the cortex, I wondered if excess Par-1 at the basolateral membranes could be explained by reduced phosphorylation at this site. With this in mind, I next examined the levels of Par-1 phosphorylation at this site in *lkb1* retinas. I found that despite the accumulation of Par-1 protein in *lkb1* retinas, the total levels of Par-1 phosphorylation at this site were much reduced, providing a possible mechanistic basis for the stabilisation and increased association of Par-1 at the membranes.

However, although DaPKC shows reduced accumulation at the sub-apical region, western analysis shows that total levels of DaPKC are unchanged; therefore it is difficult to explain why Par-1 shows reduced levels of phosphorylation. One explanation may be that Lkb1 phosphorylates DaPKC to activate it, and loss of Lkb1 leads to a kinase inactive DaPKC that is unable to maintain the lateral exclusion mechanisms that are required to keep epithelial polarity intact. I was unable to show that Lkb1 could phosphorylate DaPKC in an *in vitro* context, but this does not preclude *in vivo* regulation of DaPKC by Lkb1.

In addition, Lkb1 itself may also be responsible for the phosphorylation and activation of Par-1 itself at this motif. Previous reports have demonstrated that human LKB1 can phosphorylate human Par-1 homologues, and that phosphorylation on these motifs were required for Par-1A activity (Lizcano et al., 2004; Spicer et al., 2003), and a very recent paper by Wang *et al* has demonstrated that *Drosophila* Lkb1 can phosphorylate Par-1, albeit on a different site (Wang et al., 2007a).

This result demonstrating reduced phosphorylation at a regulatory site was interesting since it suggests 2 possibilities: 1. That Par-1 may be stabilised because increased association at the membrane protects it from degradation 2. Since this phosphorylation event also inhibits the kinase activity of Par-1, that stabilised Par-1 may be activated at the membrane.

One piece of data that suggests that Par-1 may instead be activated in *lkb1* clones was presented by Bayraktar *et al*. They showed that while loss of Par-1 in the imaginal eye disc did not exhibit polarity defects, overexpression of Par-1 could lead to severe defects in epithelial polarity. They further

demonstrated that overexpressed Par-1 was also required to be kinase active, since kinase dead versions of Par-1 that were overexpressed failed to show defects in polarity.

This data suggests two distinct possibilities; firstly these results lend weight to the possibility that stabilised Par-1 in *lkb1* clones is also activated, since *lkb1* clones also exhibit severe defects in epithelial polarity. Secondly, an alternative explanation for the lack of polarity defects in the eye disc, as suggested by Bayraktar *et al,* is that Par-1 may have a redundant or maintenance function in epithelial polarity in the eye disc. As with Lkb1, Par-1 may instead have a necessary role later during pupal development, and indeed, data from this study demonstrating morphogenetic and localisation defects during pupal development and in the adult eye support such a model.

Further data that may also support a model where Par-1 is activated in *lkb1* clones are the contrasting mislocalisation patterns of DaPKC and Baz. The ectopic expression of Baz has been shown to lead to the formation of ectopic Par complexes (Petronczki and Knoblich, 2001). Baz expression in *lkb1* clones include mislocalisation from the AJs, as well as distinct puncta near the basolateral membrane. In contrast DaPKC in *lkb1* clones often appears reduced in concentration at the apical domain and is very infrequently observed in distinct puncta; DaPKC mislocalisation appears instead as a diffuse expression, suggesting that DaPKC is not recruited by ectopic Baz. Par-1 has been demonstrated to restrict the formation of apical complexes at the basolateral domain; by creating 14-3-3 sites on Baz, Par-1 prevents the formation of a functional apical complex, since Baz that is bound to 14-3-3 cannot bind to DaPKC (Benton and St Johnston, 2003a; Benton and St

Johnston, 2003b). Hence excess levels of active Par-1 may affect the appropriate formation of apical complexes, and lead to the defects observed in aPKC and Baz localisation.

However, an alternative model and possible explanation for contrasting expression patterns of DaPKC and Baz may be that Lkb1 itself may somehow function to positively regulate the Baz-DaPKC interaction; indeed one such mechanism may be through the phospho-modification of Baz, which I demonstrated could occur *in vitro*.

Wang *et al* demonstrated that Lkb1 mediated phosphorylation of Par-1 could lead to the activation of Par-1. This recent data is consistent with the results of the genetic interaction assay, which suggest that Lkb1 and Par-1 act in the same direction. Additionally, loss of *par-1* and *lkb1* clones in the eye show phenotypic similarities, suggesting that in the absence of *lkb1*, Par-1 has reduced activity. Bayrakter *et al* showed that loss of function of *par-1* in the embryo could lead to a lateral expansion of AJs, and this is also observed in *lkb1*mutant clones in the retina. These results provide strong evidence that Lkb1 functions to positively regulate Par-1 activity, and this regulation of Par-1 activity may be conserved since mammalian studies have also shown that LKB1 can act *in vitro* to activate Par-1(Lizcano et al., 2004; Spicer et al., 2003).

Taken together, the above data weighs in favour of a model where the normal function of Lkb1 is to activate Par-1.

How might the regulation of Par-1 by Lkb1 relate to the control of epithelial polarity? The residue that is phosphorylated by aPKC in the spacer domain of Par-1 is conserved (Vaccari et al., 2005), and this phosphorylation is

necessary for restricting Par-1 from the apical membranes. It is surprising therefore, since apical regulators of Par-1 localisation show basal spreading, that Par-1 still exhibits a largely normal and polarised, albeit expanded localisation across the lateral as well as the basal domain.

Possible explanations for this are firstly, that Par-1 accumulation is upstream of the basal expansion seen by many polarity determinants, including Baz and DaPKC. This model is borne out by data from *lkb1* clones in 30% pd pupal retinas, which show normal morphology of mutant ommatidia but accumulation of Par-1, and is supported by data presented by Huynh *et al,* who showed that the localisation of Par-1 in *Drosophila* is upstream of and does not require Bazooka or Par-6 in Drosophila (Huynh et al., 2001). Secondly, Par-1 functions in a conserved lateral exclusion mechanism with members of the Par complex that results in a mutual restriction of polarity determinants to their respective domains. Par-1 phosphorylates Baz and thus prevents formation of active Par complex at the basolateral domain, since Baz has a conserved function in recruiting other members of the Par complex. Inactive Par-1 may no longer be able to inhibit the spread of the Par complex, and the loss of this mechanism may thus lead to an expansion in the expression of apical determinants. This model would further explain the severity and penetrance of defects observed in aPKC and Baz, compared to that seen with members of the SAR complex.

Since many aspects of the observed phenotype of *lkb1* clones in the fly eye have also been observed in Par-1 studies (Bayraktar et al., 2006; Martin and St Johnston, 2003; Sun et al., 2001){study, 2007 #1196}, it is indeed possible that some aspects of the *lkb1* phenotype are due to the misregulation

of Par-1. Although genetic assays have indicated that this is the case, the specific details of this question remains largely unanswered, therefore an obvious direction for future work is to determine whether Lkb1 is working through Par-1 to regulate epithelial polarity and AJ formation, and whether the *lkb1* polarity phenotype can be in part attributed to an impairment of normal Par-1 function.

Although inhibitory phosphorylation of Par-1 is decreased in *lkb1* clones, the activation status of Par-1 is unclear. An informative experiment to further explore this theory would be to determine the activation status of excess Par-1 in clones deficient for *lkb1*. Technical problems with a p-Par-1 antibody raised against the T-Loop motif precluded a clear answer, since I was unable to achieve a clear signal with this antibody. Further analysis thus could include looking at the phosphorylation status of this motif, since this site regulates the kinase activity of Par-1. Since Wang *et al* have already shown that Lkb1 phosphorylates this motif, the prediction is that Par-1 is inactivated in *lkb1* clones. Resolving this question will help us propose a mechanism and further understand the function of Lkb1.

Since both mammalian and *Drosophila* Lkb1 have been shown to phosphorylate Par-1, a conserved role for Lkb1 and Par-1 in regulating polarity in mammalian systems is probable (Lizcano et al., 2004; Wang et al., 2007a).

*Loss of Lkb1 leads to a detachment of ommatidia from the cone cell roof and defects in cone cell morphology*

At 10 and 20% pd mutant clones, I observed a defect in ommatidial positioning, whereby ommatidia appeared to be detaching from the cone cell

roof. I began my analysis of this defect by looking at ommatidial preclusters in the larval disc. I did not find any defects in ommatidial morphology, placement, or in the localisation of DN-cad or Arm in the larval disc, demonstrating that defects begin later in development.

In order to follow the development of cone cell closure over the developing PRC chamber in mutant clones, I next examined *lkb1* clones at 10% pd. Again, I was unable to detect defects in Arm distribution or in cone cell formation, suggesting that mutant ommatidia were able to remodel their junctions normally at this early stage of development to enable the apical displacement of cone cells. However, I did find that ommatidia had started detaching from the epithelium at this stage, suggesting either that the detachment of mutant ommatidia from the cone cell roof occurs through the impairment of a different mechanism than the process that lead to cone cell defects, or more likely, that the loss of contacts with PRCs destabilise the cone cell clusters shortly after. It has been demonstrated that cone cells stabilise underlying PRC clusters, by the formation of junctions between specific PRCs in the ommatidia (Longley and Ready, 1995). It is therefore possible that the loss of these contacts could in turn lead to the destabilisation of the cone cell roof, and result in defects in cone cell morphology.

Prominent AJs anchor the distal and proximal tips of the rhabdomeres to cone cells. These are known as 'rhabdomere tip junctions' (Tepass and Harris, 2007). These mediate a connection of the proximal and distal tips of the rhabdomeres to the cone cell roof, that when lost, may result in the loss of *lkb1* ommatidia from the plane of the epithelium. Examination of these junctions in *lkb1* clones may yield further insight into the morphological defects observed

in cone cells, and into the defects in ommatidial attachment to the cone cell plate.

Additionally, defects in actin were evident in 10% pd clones, suggesting that a loss of actin localisation at the apical membrane of the epithelia may contribute to, or cause defects in the attachment of ommatidia to the cone cell roof. *lkb1* ommatidia appear longer and less defined in relation to their wildtype neighbours further suggesting that defects in actin localisation may contribute to defects in ommatidial and PRC morphology. Certainly, defects in PRC shape were observed at 10 and 20% pd that may be attributed to either defective junctions, or defects in the actin cytoskeleton, since both have essential roles in the maintenance of cell shape.

I next examined the cone cells at 17 and 20% pd, and found that cone cells morphology in *lkb1* clones was severely disrupted. Cone cells were often more numerous; while the normal number of cone cells in a group is four, I frequently observed five or more cells. In addition, cone cells were also occasionally missing, suggesting ectopic cell death. Finally, I sometimes observed a fusion of two or more cone cell groups, with more than two primary pigment cells, possibly derived from more than one cone cell cluster. DN-cad and Arm expression appeared to be mostly normal in these cells, although DN-cad occasionally appeared as a closed circle of expression within the cone cells, rather than a semi circle, suggesting ectopic localisation.

Hayashi *et al* showed that *Roi* mutants, despite having a variable number of cone cells per group, continued to adhere to the predicted stable configurations, and cadherins were shown to play an essential role in the development and pattern formation of cone cells. Cone cells mutant for *DE-*

*cad* were shown to dissociate from other cone cells in the same group. In contrast, *lkb1* mutant cone cells occasionally aggregate into groups containing more than four cone cells, and in addition the fusion of distinct cone cell groups can also sometimes be observed. This suggests that cone cells mutant for *lkb1* may not be lacking adhesion between cells, but may instead be experiencing increased adhesion between cone cell groups.

Cone cell configuration, and the differential adhesive properties of equatorial and polar cone cells versus the anterior and posterior cone cells are determined by cadherins, however, there appeared to be no loss or severe defects in DN-cad expression in *lkb1* cone cells. In addition, Arm also looked normal in distribution and levels in mutant cone cells.

Longley *et al* showed that integrins were required to maintain the retinal floor, and that $mys^{nj42}$ mutants displayed a similar phenotype later in pupal development, at 55% pd, where the R8 cell body appears to fall through the retinal floor (Longley and Ready, 1995). However, Longley showed that normal integrin function was not required until approx 37% pd, suggesting that defects in integrin accumulation at the cone cell plate are not involved in the defects in *lkb1* ommatidial positioning in the epithelium.

Bao *et al* carried out a study of patterning in the pupal retina, and showed that the *Drosophila* immunoglobin superfamily members Hibris and Roughest interactions promote junction and pattern formation in the retina (Bao and Cagan, 2005). They described a model where retinal cells could exhibit preferential binding to primary cells on the basis of differential expression patterns of pattern determinants Hibris and Roughest. They also showed that different Roughest and Hibris expression patterns could lead to

cells competing for particular cell fates. Cone cells deficient for *lkb1* exhibit defects in binding, as well as an excess number of cone cells or accessory cells within a cluster, suggesting possible defects in the cell fate process. Thus examining the expression patterns of determinants such as Roughest and Hibris with established functions in cone cell development may yield to further insight into the mechanisms by which Lkb1 normally functions to regulate cone cell formation.

The junctions are particularly important in the morphogenesis of the pupal eye, since AJs between the PRCs aid in cell shape changes, as well as in keeping the early PRC cluster intact as the apical surfaces of the larval PRCs begin to displace to face the centre of the clusters in early pupal life. In addition, AJs serve to stabilise and support PRCs during the elongation process after 55% pd of pupal development. It is therefore intriguing, yet perplexing that *lkb1* ommatidia are able to complete the 90° shift in orientation early in pupal life, as well as extend appropriately in the proximo-distal axis, yet display defects in attachment to the cone cell roof, and in AJ structure in mid pupal retinas. It is likely that mutant retinas have the ability to recover from this defect, in a similar way to adult *crb* mutants that show recovery in ZA formation, despite severe defects observed earlier during pupal development (Pellikka et al., 2002).

Further analysis of this phenotype will also involve looking at the arrangement of cone cell groups in mutant epithelia; cone cells that do not conform to a limited set of stable configurations suggest a defect in adhesive processes.

I have presented data that suggests a critical role for Lkb1 during morphogenetic processes. Further work will include continuing to characterise these defects, and identifying mechanisms and biochemical interactions between Lkb1 and other polarity determinants. One particularly intriguing question that remains unanswered is whether the role of Lkb1 in regulating epithelial polarity and junction formation is restricted to specific periods of morphogenesis or whether Lkb1 has a more generalised role throughout development in the maintenance of polarity. The data presented in this chapter, together with that presented in chapter 4 may suggest roles for Lkb1 that can be delineated by specific developmental stages.

# 6 General Conclusions

During the course of these studies, I set out to investigate the biological function and roles of Lkb1. I set out to do this in two ways; firstly, by conducting a genetic screen to identify biological interactors of Lkb1, and secondly, by characterising the loss of function phenotype of *lkb1* in the *Drosophila* eye.

Our modifier screen of approximately 9,500 flies yielded 26 mutants, which have been rigorously tested for specificity and reproducibility. These are now ready to be mapped.

The primary focus of this thesis was to describe and characterise the polarity phenotype of Lkb1 in the *Drosophila* eye. Data from the *Drosophila* oocyte and mammalian intestinal cell lines has suggested that Lkb1 has a role in the initial generation of polarity. This study further indicates an essential role for Lkb1 in the maintenance of epithelial polarity in *Drosophila* pupal retinas, based on the following results.

I have presented evidence that in the absence of Lkb1, apical, sub-apical, and junctional proteins exhibit an expansion of expression, and I have shown that markers for the different membrane domains overlap, suggesting that membrane identity is compromised in *lkb1* clones. Key polarity determinants are often observed at ectopic locations in *lkb1* PRCs, with the Par complex proteins, Baz and DaPKC in particular demonstrating consistent mislocalisation defects. Strikingly, these defects were seen in tandem with morphogenetic defects, strongly suggesting that the mislocalisation of Baz and

DaPKC may be precipitating factors for the aberrant morphogenesis of mutant PRCs.

Co-localisation studies with the above polarity markers and Arm revealed that mislocalisation of Arm was also consistently associated with defects in PRC morphology, and defects in Arm could be observed very early in pupal development around the onset of morphogenetic defects, suggesting that the mislocalisation of Arm is also a primary defect in *lkb1* clones.

Immuno-fluorescence and immuno-blotting experiments revealed that Arm and Par-1 are stabilised in the absence of Lkb1, and may provide a mechanistic basis for the defects in epithelial polarity observed in *lkb1* tissue. However, subsequent experiments by colleagues in my lab to replicate the finding that Arm protein levels were stabilised in *lkb1* mutant tissue have yielded ambiguous results, and efforts to clarify this are ongoing.

One of the hallmarks of elevated Wg signalling is a stabilisation of Arm, and previous data has implicated Lkb1 in the control of Wnt signalling, however, I did not find evidence that Wg signalling was altered in *lkb1* clones, suggesting that Arm, if indeed stabilised, may be stabilised via a different mechanism.

Although Par-1 levels were elevated in *lkb1* clones, I found that the relative levels of phospho-Par-1 were greatly reduced. This phospho-modification of Par-1 is inhibitory, and serves to regulate the activity and localisation of Par-1; decreased levels of Par-1 phosphorylation suggest that the excess Par-1 in *lkb1* clones may preferentially associate with the cortex, and that its kinase function may also be activated. However, recent work by

Wang *et al* reveals that, conversely, Lkb1 has a role in the activation of Par-1, through phosphorylation of its activation motif.

Indeed genetic interaction data has suggested a complex interaction of Lkb1 with both Arm and Par-1. Further work is required to reconcile and clarify these interactions, and to test whether the mislocalisation of Arm and the accumulation of Par-1 are responsible for part of the morphogenetic defects seen in *lkb1* clones. In particular, establishing whether excess Arm does indeed accumulate in *lkb1* mutant tissue may help to explain how Lkb1 regulates the formation of AJ and Armadillo localisation.

Another key finding was that Lkb1 also affects the structure and formation of adherens junctions. AJs are thought to play central roles in the establishment and maintenance of epithelial polarity, (reviewed in (Nelson, 2003)), and the segregation of basolateral cues (Harris and Peifer, 2004); as such Lkb1 mediated regulation of AJs may be crucial to its function in the maintenance of epithelial polarity.

The establishment and maintenance of polarity involves a number of cues: in addition to landmark positioning such as adherens junctions, cortical landmarks (including Par proteins), cytoskeletal cues and membrane trafficking are also integral to the establishment and maintenance of polarity. Results from this study suggest that Lkb1 is involved in regulating at least three of these processes, since in addition to defects observed in AJ formation and the localisation of cortical cues, *lkb1* clones also exhibit defects in the localisation of actin in ommatidia, beginning early in pupal development.

Data from this study, together with previous work by Martin *et al* demonstrating the role of Lkb1 in the development of early A-P polarity in the

oocyte, and epithelial polarity in follicle cells (Martin and St Johnston, 2003), indicate a general and potentially central role for Lkb1 in the development and maintenance of different classes of cell polarity. In addition, results from this study suggest distinct roles for Lkb1 during specific developmental and morphogenetic periods.

Key elements of these polarity-generating systems are highly conserved, from flies to humans, suggesting that Lkb1 specific interactions observed in the *Drosophila* retina may also be conserved in mammalian epithelia.

Conserved interactions between Lkb1 and Par-1 have already been demonstrated, therefore, it is likely that human LKB1 functions in similar ways to regulate Par-1 and epithelial polarity. In addition, work by Lin-Marq *et al* showed that LKB1 could induce the phosphorylation of β-catenin (Lin-Marq et al., 2005), and Back *et al* showed that β-catenin could be redistributed to the nucleus in PJS polyps (Back et al., 1999), suggesting that misregulation of β-catenin may also occur in PJS, although the second findings were inconclusive since the misregulation of β-catenin was not replicated in other findings (Herter et al., 1999).

Future work will concentrate on establishing a mechanistic basis for Lkb1 mediated control of cell polarity. *In vitro* kinase experiments showed that some polarity determinants could act as substrates for Lkb1, and further exploration of this, both *in vitro* and *in vivo* will be informative. In particular, the biochemical basis for the stabilisation of Par-1 and possibly of Arm may shed further light on this question.

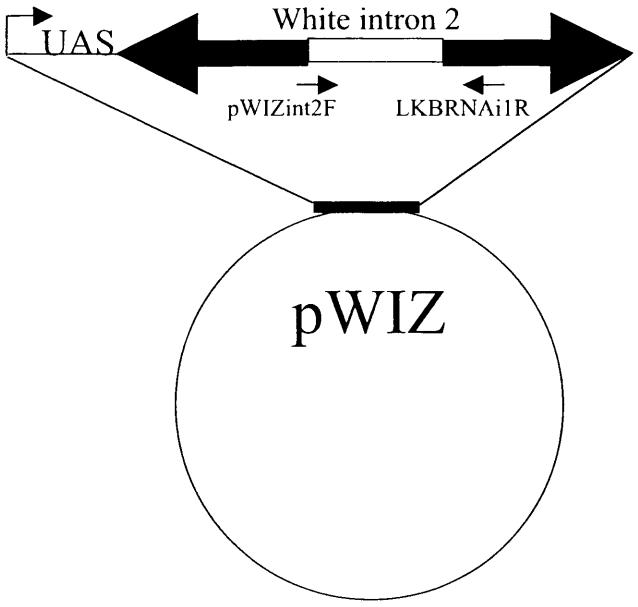
The intimate connection between the regulation of polarity and proliferation has been demonstrated in many studies. Thus, the described role

for Lkb1 in regulating polarity may prove to be an important basis for understanding the mechanism of tumorigenesis in PJS.

# 7 Appendices

## 7.1 Appendix 1.1: *pWiz[UAS-Lkb1-RNAi]* Lkb1 hairpin sequence

### 7.1.1 Vector Map *pWiz[UAS]*




This vector contains an intron from the white gene flanked by two MCS. Cloning of the target DNA upstream and downstream of the intron allows the generation of a construct with two inversely oriented pieces of identical DNA separated by an intron. The resultant RNA makes a fold back construct with an intervening single stranded intron that is spliced yielding dsRNA (generated in the R. Carthew lab).

### 7.1.2 Lkb1 RNAi hairpin sequence

30-

GGTAAAGCAGCCGGTGGAGCAGAAGCAGGAGGGGCAGGAGCAACAGGCCAGAT

GACTGTGACCACCATGGAGGCACAGGTCAAGGCA**GCACACCATCATCATCTAC**

ACCATCCCACGGGCGGA**GGGGCCCAGCACAAGGTGG**AGGAGGAGGAGCCGGAT

CCTGTTGAGGACGAGATGACCGTACTGCTGGCCA**ATAAGAACTTTCACTATGA**

**CGTCGCC**TCCGATCTGGACGACGACGCTTTTGTGGAGCTCA-283

### 7.1.3 Lkb1 RNAi Primers

Lkb-RNAi primers with Xba sites at each end were used to clone the 250bp fragment of Lkb1 into the MCS in the pWiz construct:

Forward Primer: 5'-GTTCTAGATCTCGGCCAGTCGGA-3'

Reverse Primer: 5'-ATTCTAGACTTGGGCGGTGTCTTCTT-3'

Bold characters in the Lkb1 RNAi sequence above are the primers used for the sequencing and verification of correctly oriented inserts – P1, P2 and P3

## 7.2 Appendix 1.2: Lkb1 constructs for recombinant protein and Lkb1 antibody production

The Lkb1 coding sequence was amplified from a *Drosophila* embryo cDNA library.

### 7.2.1 The Lkb1 coding sequence

1-GTTATTCCAGCGTTCGTCCCGAAAAATACAAATTTATTATTTTTCGTTAA

TGTTTTTTTTTTTCAGCAGCGACAGTAGCAACAACAACCAGTGCTCGTGC

AGCCCTGGTTGCCCGCGACCAGTGTGACCGTATTGCGGCGAACTTACTTT

GGCCAAAAACTAATTGTTAAACAATTTTTCGGAAGGGGAAAAGAACAAAT

TGAACGGCGAGAGTAAACACAGGAATTCACAAATTTCTATGAGAAACAGA

TCGCACGACGGTGAAACGAAAGGGGGGAACGCCTTTCCAGCGGACATCGC

AGAATAGACGACGGGCGACGAGGGACCTCGAAAAATCGCACGGCAATCAA

TTCGAGTGTGTGCGAATTGATTACAAAAATAAAGGTATTTCGAGAGCCAC

AGGCAACACAGGGACCACACCGTCCACTCCCATCCGCCGGAGTGGGGCTG

CCACCTGCGATATGTGATCATTGGGGCTCCGCGGAGGTTTATGCAATGTT

CTAGCTCTCGGCCAGTCGGAGGTAAAGCAGCCGGTGGAGCAGAAGCAGGA

GGGGCAGGAGCAACAGGCCAGATGACTGTGACCACCATGGAGGCACAGGT

CAAGGCAGCACACCATCATCATCTACATCATCCCACGGGCGGAGGGGCCC

AGCACAAGGTGGAGGAGGAGGAGCCGGATCCTGTTGAGGACGAGATGACC

GTACTGCTGGCCAATAAGAACTTTCACTATGACGTCGCCTCCGATCTGGA

CGACGACGCTTTTGTGGAGCTCAAAGAAGACACCGCCCAAGCGGATGATG

CGGGCGCGGGCGTTGGCTTCTACAATCCGGATGAACTGCTGCTCGATGAC

CAGCCACATCCGCAAGTCACGTGGCTGGACGACGACGAGATCGAGACGCT

CGATCGCGTGACGCTGGACATGGGTAATATGTTCTTCAATCGCGTCGACA

GCCAGGACATCATCTATCAGCAGAAAAAGAAGAGCATTAAGATGGTGGGC

AAGTACATAATGGGCGATGTTCTCGGCGAGGGATCGTACGGCAAGGTGAA

GGAGGCCATGAACTCGGAGAACCTGTGCCGCCTGGCCGTCAAGATCCTGA

CTAAGCGGAAGTTGCGCCGGATTCCCAACGGCGAACAGAACGTAACGCGC

GAGATCGCCTTGCTGAAGCAGCTGAAGCATCGACATGTCGTGGAGCTGGT

CGATGTTTTGTACAACGAGGAGAAGCAGAAAATGTACTTGGTCATGGAGT

ACTGTGTCGGCGGGCTGCAGGAGATGATTGACTATCAGCCAGACAAGCGG

ATGCCGCTGTTTCAGGCGCACGGTTACTTCAAACAGCTAGTCGACGGCCT

GGAGTACTTGCACAGCTGCCGGGTCATCCACAAGGATATCAAGCCAGGCA

ACCTGCTGCTCTCCCTGGATCAAACGCTGAAGATATCCGACTTCGGTGTG

GCGGAGCAACTGGATCTGTTCGCACCTGACGACACATGCACGACGGGCCA

AGGTTCTCCGGCCTTCCAGCCACCGGAGATTGCCAACGGACACGAGACGT

TCGCCGGCTTCAAGGTGGACATCTGGTCCAGCGGAGTGACTCTCTACAAT

TTGGCCACTGGACAGTATCCCTTCGAGGGCGACAATATCTACCGACTGCT

GGAGAACATCGGGCGAGGTCAGTGGGAGGCGCCCGCGTGGCTATACGAAA

TGGACGCGGACTTTGCCAACCTGATTCTCGGCATGCTTCAGGCTGATCCC

AGTAAACGCCTCTCTCTGCAGGAAATACGCCACGACACTTGGTTCCGATC

CGCACCGGTTAAGACCGGCCCACCGATACCCATTCCCCCCCTGAAGGGTG

ACAAATACCGCAACTCCACGGTGATACCTTACTTGGAAGCTTACCACTAC

GGCACCCAGGAGGAGGATGTCTACTTTACAGAACACGACGTAAATCAGGA

GCTCGCCCGCCAAGCGGCAGCTGCTGCCTCCGAAATTCGGGCCAAACAGT

CGGCGGCAGCCCTAGCCGCCTGCCACACCTACGAACCGCCCTCCACAAGT

GCCGCAGCCGCCAGCAATTCGCTGGGCAACGGTAGCAGAGAGGAGGCGCC

CGTCAAGAAGAAGGGATCGGCACTGAAGAGGCGCGCCAAGAAGCTGACGT

CCTGCATCTCCGTGCGCAAGCTGAGCCACTGCCGAACTTCGTAGGCGCCT

TAGATGCCTCCACTAAACATAGATGCTAAGATTTATCGAAAGGAATAACT

TTGTTAACAAATTAATCATGTAGGAAATTCAGGCGCAACCTCGTACCGCC

CGAGCAGCAGGAGCAGCAGCGGTCGCATCAACAGCAGCAACAGCCCGCAT

CAGCGGAGTTGGCTGTGGAATTGGGGAGCTATGGGAGCGACACGAAAGCT

ATATATCCATGTGACAATACTGTGTATTATATGAAACAAATGAATGCGTA

AATTAGTCGTTAATATATTATGATGTACTGAAGGCAGCCGAGGATTGCCG

GAGGAGACGGTGCCCATTGGTGCGTCGCACACATAAAGAAATATGTTCAA

GTGAAATT-2558

## 7.2.2 Lkb1 primers

### *7.2.2.1 pBAD/Thio TOPO-Lkb1*

The Lkb1 coding sequence was cloned into the pBAD/Thio TOPO vector (Invitrogen) using the following primers:

Forward Primer: 5'-ATGCAATGTTCTAGCTCTCGGCCAG-3'

Reverse Primer 5'-CGAAGTTCGGCAGTGGCTCA-3'

### *7.2.2.2 pGEX-4T-1-Lkb1*

The Lkb1 coding sequence was cloned into the pGEX vector MCS (GE Healthcare Life Sciences):

Forward Primer with EcoR1 site:

5'-CATGGAATTCATGCAATGTTCTAGCT-3'

Reverse Primer with Xho1 site:

5'-CGCTCGAGCGAAGTTCGG-3'

### *7.2.2.3 pMAL-c2X-Lkb1*

Lkb1 was cloned into pMAL-Cri (New England Biolabs) using the following primers:

Forward Primer: 5'-ATGCAATGTTCTAGCTCTCGGCCAG-3'

Reverse Primer 5'-CGAAGTTCGGCAGTGGCTCA-3

## References

**Abdelilah-Seyfried, S., Cox, D. N., and Jan, Y. N.** (2003). Bazooka is a permissive factor for the invasive behavior of discs large tumor cells in Drosophila ovarian follicular epithelia. *Development* 130, 1927-1935.

**Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R.** (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *Embo J* 16, 3797-3804.

**Adams, C. L., Nelson, W. J., and Smith, S. J.** (1996). Quantitative analysis of cadherin-catenin-actin reorganization during development of cell-cell adhesion. *J Cell Biol* 135, 1899-1911.

**Ahmed, Y., Hayashi, S., Levine, A., and Wieschaus, E.** (1998). Regulation of armadillo by a Drosophila APC inhibits neuronal apoptosis during retinal development. *Cell* 93, 1171-1182.

**Amit, S., Hatzubai, A., Birman, Y., Andersen, J. S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y., and Alkalay, I.** (2002). Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes Dev* 16, 1066-1076.

**Baas, A. F., Boudeau, J., Sapkota, G. P., Smit, L., Medema, R., Morrice, N. A., Alessi, D. R., and Clevers, H. C.** (2003). Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD. *Embo J* 22, 3062-3072.

**Baas, A. F., Kuipers, J., van der Wel, N. N., Batlle, E., Koerten, H. K., Peters, P. J., and Clevers, H. C.** (2004). Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD. *Cell* 116, 457-466.

**Bachmann, A., Schneider, M., Theilenberg, E., Grawe, F., and Knust, E.** (2001). Drosophila Stardust is a partner of Crumbs in the control of epithelial cell polarity. *Nature* 414, 638-643.

**Back, W., Loff, S., Jenne, D., and Bleyl, U.** (1999). Immunolocalization of beta catenin in intestinal polyps of Peutz-Jeghers and juvenile polyposis syndromes. *J Clin Pathol* 52, 345-349.
**Bao, S., and Cagan, R.** (2005). Preferential adhesion mediated by Hibris and Roughest regulates morphogenesis and patterning in the Drosophila eye. *Dev Cell* 8, 925-935.
**Bardeesy, N., Sinha, M., Hezel, A. F., Signoretti, S., Hathaway, N. A., Sharpless, N. E., Loda, M., Carrasco, D. R., and DePinho, R. A.** (2002). Loss of the Lkb1 tumour suppressor provokes intestinal polyposis but resistance to transformation. *Nature* 419, 162-167.
**Bayraktar, J., Zygmunt, D., and Carthew, R. W.** (2006). Par-1 kinase establishes cell polarity and functions in Notch signaling in the Drosophila embryo. *J Cell Sci* 119, 711-721.
**Benton, R., and St Johnston, D.** (2003a). A conserved oligomerization domain in drosophila Bazooka/PAR-3 is important for apical localization and epithelial polarity. *Curr Biol* 13, 1330-1334.
**Benton, R., and St Johnston, D.** (2003b). Drosophila PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. *Cell* 115, 691-704.
**Berger, J., Suzuki, T., Senti, K. A., Stubbs, J., Schaffner, G., and Dickson, B. J.** (2001). Genetic mapping with SNP markers in Drosophila. *Nat Genet* 29, 475-481.
**Beronja, S., Laprise, P., Papoulas, O., Pellikka, M., Sisson, J., and Tepass, U.** (2005). Essential function of Drosophila Sec6 in apical exocytosis of epithelial photoreceptor cells. *J Cell Biol* 169, 635-646.
**Betschinger, J., Mechtler, K., and Knoblich, J. A.** (2003). The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. *Nature* 422, 326-330.
**Bilder, D., Li, M., and Perrimon, N.** (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* 289, 113-116.
**Bohm, H., Brinkmann, V., Drab, M., Henske, A., and Kurzchalia, T. V.** (1997). Mammalian homologues of C. elegans PAR-1 are asymmetrically localized in epithelial cells and may influence their polarity. *Curr Biol* 7, 603-606.
**Boudeau, J., Baas, A. F., Deak, M., Morrice, N. A., Kieloch, A., Schutkowski, M., Prescott, A. R., Clevers, H. C., and Alessi, D. R.** (2003). MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. *Embo J* 22, 5102-5114.
**Brajenovic, M., Joberty, G., Kuster, B., Bouwmeester, T., and Drewes, G.** (2004). Comprehensive proteomic analysis of human Par protein complexes reveals an interconnected protein network. *J Biol Chem* 279, 12804-12811.
**Cadigan, K. M., Jou, A. D., and Nusse, R.** (2002). Wingless blocks bristle formation and morphogenetic furrow progression in the eye through repression of Daughterless. *Development* 129, 3393-3402.
**Cadigan, K. M., and Nusse, R.** (1996). wingless signaling in the Drosophila eye and embryonic epidermis. *Development* 122, 2801-2812.
**Cagan, R. L., and Ready, D. F.** (1989). The emergence of order in the Drosophila pupal retina. *Dev Biol* 136, 346-362.
**Chalmers, A. D., Pambos, M., Mason, J., Lang, S., Wylie, C., and Papalopulu, N.** (2005). aPKC, Crumbs3 and Lgl2 control apicobasal polarity in early vertebrate development. *Development* 132, 977-986.
**Chanut, F., and Heberlein, U.** (1995). Role of the morphogenetic furrow in establishing polarity in the Drosophila eye. *Development* 121, 4085-4094.
**Collins, S. P., Reoma, J. L., Gamm, D. M., and Uhler, M. D.** (2000). LKB1, a novel serine/threonine protein kinase and potential tumour suppressor, is phosphorylated by cAMP-dependent protein kinase (PKA) and prenylated in vivo. *Biochem J* 345 Pt 3, 673-680.
**Cox, D. N., Seyfried, S. A., Jan, L. Y., and Jan, Y. N.** (2001). Bazooka and atypical protein kinase C are required to regulate oocyte differentiation in the Drosophila ovary. *Proc Natl Acad Sci U S A* 98, 14475-14480.
**Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A.** (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785-789.
**den Hollander, A. I., Johnson, K., de Kok, Y. J., Klebes, A., Brunner, H. G., Knust, E., and Cremers, F. P.** (2001). CRB1 has a cytoplasmic domain that is functionally conserved between human and Drosophila. *Hum Mol Genet* 10, 2767-2773.

**Djiane, A., Yogev, S., and Mlodzik, M.** (2005). The apical determinants aPKC and dPatj regulate Frizzled-dependent planar cell polarity in the Drosophila eye. *Cell* 121, 621-631.
**Doerflinger, H., Benton, R., Torres, I. L., Zwart, M. F., and St Johnston, D.** (2006). Drosophila anterior-posterior polarity requires actin-dependent PAR-1 recruitment to the oocyte posterior. *Curr Biol* 16, 1090-1095.
**Drubin, D. G.** (2000). Cell Polarity, Oxford City Press).
**Fernandez, P., Carretero, J., Medina, P. P., Jimenez, A. I., Rodriguez-Perales, S., Paz, M. F., Cigudosa, J. C., Esteller, M., Lombardia, L., Morente, M., *et al.*** (2004). Distinctive gene expression of human lung adenocarcinomas carrying LKB1 mutations. *Oncogene* 23, 5084-5091.
**Fujioka, M., Jaynes, J. B., Bejsovec, A., and Weir, M.** (2000). Production of transgenic Drosophila. *Methods Mol Biol* 136, 353-363.
**Ghaffar, H., Sahin, F., Sanchez-Cepedes, M., Su, G. H., Zahurak, M., Sidransky, D., and Westra, W. H.** (2003). LKB1 protein expression in the evolution of glandular neoplasia of the lung. *Clin Cancer Res* 9, 2998-3003.
**Giardiello, F. M., Welsh, S. B., Hamilton, S. R., Offerhaus, G. J., Gittelsohn, A. M., Booker, S. V., Krush, A. J., Yardley, J. H., and Luk, G. D.** (1987). Increased risk of cancer in the Peutz-Jeghers syndrome. *N Engl J Med* 316, 1511-1514.
**Golic, K. G.** (1991). Site-specific recombination between homologous chromosomes in Drosophila. *Science* 252, 958-961.
**Golic, K. G., and Lindquist, S.** (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. *Cell* 59, 499-509.
**Gottardi, C. J., Wong, E., and Gumbiner, B. M.** (2001). E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. *J Cell Biol* 153, 1049-1060.
**Grawe, F., Wodarz, A., Lee, B., Knust, E., and Skaer, H.** (1996). The Drosophila genes crumbs and stardust are involved in the biogenesis of adherens junctions. *Development* 122, 951-959.
**Guo, S., and Kemphues, K. J.** (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* 81, 611-620.
**Gustafson, K., and Boulianne, G. L.** (1996). Distinct expression patterns detected within individual tissues by the GAL4 enhancer trap technique. *Genome* 39, 174-182.
**Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., Harms, M., Nardine, T., Luo, L., and Dickson, B. J.** (2002). Rac function and regulation during Drosophila development. *Nature* 416, 438-442.
**Harris, T. J., and Peifer, M.** (2004). Adherens junction-dependent and -independent steps in the establishment of epithelial cell polarity in Drosophila. *J Cell Biol* 167, 135-147.
**Harris, T. J., and Peifer, M.** (2005). The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. *J Cell Biol* 170, 813-823.
**Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R., and Hardie, D. G.** (2003). Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* 2, 28.
**Hayashi, T., and Carthew, R. W.** (2004). Surface mechanics mediate pattern formation in the developing retina. *Nature* 431, 647-652.
**Hearle, N., Schumacher, V., Menko, F. H., Olschwang, S., Boardman, L. A., Gille, J. J., Keller, J. J., Westerman, A. M., Scott, R. J., Lim, W., *et al.*** (2006). Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. *Clin Cancer Res* 12, 3209-3215.
**Heberlein, U., Singh, C. M., Luk, A. Y., and Donohoe, T. J.** (1995). Growth and differentiation in the Drosophila eye coordinated by hedgehog. *Nature* 373, 709-711.
**Herter, P., Kuhnen, C., Muller, K. M., Wittinghofer, A., and Muller, O.** (1999). Intracellular distribution of beta-catenin in colorectal adenomas, carcinomas and Peutz-Jeghers polyps. *J Cancer Res Clin Oncol* 125, 297-304.
**Higashijima, S., Kojima, T., Michiue, T., Ishimaru, S., Emori, Y., and Saigo, K.** (1992). Dual Bar homeo box genes of Drosophila required in two photoreceptor cells, R1 and R6, and primary pigment cells for normal eye development. *Genes Dev* 6, 50-60.
**Hong, S. P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M.** (2003a). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc Natl Acad Sci U S A* 100, 8839-8843.

**Hong, Y., Ackerman, L., Jan, L. Y., and Jan, Y. N.** (2003b). Distinct roles of Bazooka and Stardust in the specification of Drosophila photoreceptor membrane architecture. *Proc Natl Acad Sci U S A* 100, 12712-12717.
**Hong, Y., Stronach, B., Perrimon, N., Jan, L. Y., and Jan, Y. N.** (2001). Drosophila Stardust interacts with Crumbs to control polarity of epithelia but not neuroblasts. *Nature* 414, 634-638.
**Hurov, J. B., Watkins, J. L., and Piwnica-Worms, H.** (2004). Atypical PKC phosphorylates PAR-1 kinases to regulate localization and activity. *Curr Biol* 14, 736-741.
**Hutterer, A., Betschinger, J., Petronczki, M., and Knoblich, J. A.** (2004). Sequential roles of Cdc42, Par-6, aPKC, and Lgl in the establishment of epithelial polarity during Drosophila embryogenesis. *Dev Cell* 6, 845-854.
**Huynh, J. R., Petronczki, M., Knoblich, J. A., and St Johnston, D.** (2001). Bazooka and PAR-6 are required with PAR-1 for the maintenance of oocyte fate in Drosophila. *Curr Biol* 11, 901-906.
**Izaddoost, S., Nam, S. C., Bhat, M. A., Bellen, H. J., and Choi, K. W.** (2002). Drosophila Crumbs is a positional cue in photoreceptor adherens junctions and rhabdomeres. *Nature* 416, 178-183.
**Izumi, Y., Hirose, T., Tamai, Y., Hirai, S., Nagashima, Y., Fujimoto, T., Tabuse, Y., Kemphues, K. J., and Ohno, S.** (1998). An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of Caenorhabditis elegans polarity protein PAR-3. *J Cell Biol* 143, 95-106.
**Jimenez, A. I., Fernandez, P., Dominguez, O., Dopazo, A., and Sanchez-Cespedes, M.** (2003). Growth and molecular profile of lung cancer cells expressing ectopic LKB1: down-regulation of the phosphatidylinositol 3'-phosphate kinase/PTEN pathway. *Cancer Res* 63, 1382-1388.
**Jishage, K., Nezu, J., Kawase, Y., Iwata, T., Watanabe, M., Miyoshi, A., Ose, A., Habu, K., Kake, T., Kamada, N., *et al.*** (2002). Role of Lkb1, the causative gene of Peutz-Jegher's syndrome, in embryogenesis and polyposis. *Proc Natl Acad Sci U S A* 99, 8903-8908.
**Karuman, P., Gozani, O., Odze, R. D., Zhou, X. C., Zhu, H., Shaw, R., Brien, T. P., Bozzuto, C. D., Ooi, D., Cantley, L. C., and Yuan, J.** (2001). The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death. *Mol Cell* 7, 1307-1319.
**Kauffmann, R. C., Li, S., Gallagher, P. A., Zhang, J., and Carthew, R. W.** (1996). Ras1 signaling and transcriptional competence in the R7 cell of Drosophila. *Genes Dev* 10, 2167-2178.
**Kempkens, O., Medina, E., Fernandez-Ballester, G., Ozuyaman, S., Le Bivic, A., Serrano, L., and Knust, E.** (2006). Computer modelling in combination with in vitro studies reveals similar binding affinities of Drosophila Crumbs for the PDZ domains of Stardust and DmPar-6. *Eur J Cell Biol* 85, 753-767.
**Kimmel, B. E., Heberlein, U., and Rubin, G. M.** (1990). The homeo domain protein rough is expressed in a subset of cells in the developing Drosophila eye where it can specify photoreceptor cell subtype. *Genes Dev* 4, 712-727.
**Kinghorn, K. J., Crowther, D. C., Sharp, L. K., Nerelius, C., Davis, R. L., Chang, H. T., Green, C., Gubb, D. C., Johansson, J., and Lomas, D. A.** (2006). Neuroserpin binds Abeta and is a neuroprotective component of amyloid plaques in Alzheimer disease. *J Biol Chem* 281, 29268-29277.
**Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., and Nakayama, K.** (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *Embo J* 18, 2401-2410.
**Klein, T. J., and Mlodzik, M.** (2005). Planar cell polarization: an emerging model points in the right direction. *Annu Rev Cell Dev Biol* 21, 155-176.
**Knust, E., Tepass, U., and Wodarz, A.** (1993). crumbs and stardust, two genes of Drosophila required for the development of epithelial cell polarity. *Dev Suppl*, 261-268.
**Kramer, H., Cagan, R. L., and Zipursky, S. L.** (1991). Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine-kinase receptor. *Nature* 352, 207-212.
**Kuchinke, U., Grawe, F., and Knust, E.** (1998). Control of spindle orientation in Drosophila by the Par-3-related PDZ-domain protein Bazooka. *Curr Biol* 8, 1357-1365.
**Kumar, J. P., and Ready, D. F.** (1995). Rhodopsin plays an essential structural role in Drosophila photoreceptor development. *Development* 121, 4359-4370.

**Kuphal, F., and Behrens, J.** (2006). E-cadherin modulates Wnt-dependent transcription in colorectal cancer cells but does not alter Wnt-independent gene expression in fibroblasts. *Exp Cell Res* 312, 457-467.
**Langevin, J., Morgan, M. J., Sibarita, J. B., Aresta, S., Murthy, M., Schwarz, T., Camonis, J., and Bellaiche, Y.** (2005). Drosophila exocyst components Sec5, Sec6, and Sec15 regulate DE-Cadherin trafficking from recycling endosomes to the plasma membrane. *Dev Cell* 9, 355-376.
**Lebovitz, R. M., Takeyasu, K., and Fambrough, D. M.** (1989). Molecular characterization and expression of the (Na+ + K+)-ATPase alpha-subunit in Drosophila melanogaster. *Embo J* 8, 193-202.
**Lee, T., and Luo, L.** (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 22, 451-461.
**Lee, Y. S., and Carthew, R. W.** (2003). Making a better RNAi vector for Drosophila: use of intron spacers. *Methods* 30, 322-329.
**Lin-Marq, N., Borel, C., and Antonarakis, S. E.** (2005). Peutz-Jeghers LKB1 mutants fail to activate GSK-3beta, preventing it from inhibiting Wnt signaling. *Mol Genet Genomics* 273, 184-196.
**Liu, X., Rubin, J. S., and Kimmel, A. R.** (2005). Rapid, Wnt-induced changes in GSK3beta associations that regulate beta-catenin stabilization are mediated by Galpha proteins. *Curr Biol* 15, 1989-1997.
**Lizcano, J. M., Goransson, O., Toth, R., Deak, M., Morrice, N. A., Boudeau, J., Hawley, S. A., Udd, L., Makela, T. P., Hardie, D. G., and Alessi, D. R.** (2004). LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *Embo J* 23, 833-843.
**Longley, R. L., Jr., and Ready, D. F.** (1995). Integrins and the development of three-dimensional structure in the Drosophila compound eye. *Dev Biol* 171, 415-433.
**Macara, I. G.** (2004). Par proteins: partners in polarization. *Curr Biol* 14, R160-162.
**Marignani, P. A., Kanai, F., and Carpenter, C. L.** (2001). LKB1 associates with Brg1 and is necessary for Brg1-induced growth arrest. *J Biol Chem* 276, 32415-32418.
**Martin, S. G., Dobi, K. C., and St Johnston, D.** (2001). A rapid method to map mutations in Drosophila. *Genome Biol* 2, RESEARCH0036.
**Martin, S. G., and St Johnston, D.** (2003). A role for Drosophila LKB1 in anterior-posterior axis formation and epithelial polarity. *Nature* 421, 379-384.
**Matsubayashi, H., Sese, S., Lee, J. S., Shirakawa, T., Iwatsubo, T., Tomita, T., and Yanagawa, S.** (2004). Biochemical characterization of the Drosophila wingless signaling pathway based on RNA interference. *Mol Cell Biol* 24, 2012-2024.
**Michel, D., Arsanto, J. P., Massey-Harroche, D., Beclin, C., Wijnholds, J., and Le Bivic, A.** (2005). PATJ connects and stabilizes apical and lateral components of tight junctions in human intestinal cells. *J Cell Sci* 118, 4049-4057.
**Miller, D. T., and Cagan, R. L.** (1998). Local induction of patterning and programmed cell death in the developing Drosophila retina. *Development* 125, 2327-2335.
**Mollereau, B., Dominguez, M., Webel, R., Colley, N. J., Keung, B., de Celis, J. F., and Desplan, C.** (2001). Two-step process for photoreceptor formation in Drosophila. *Nature* 412, 911-913.
**Morata, G., and Ripoll, P.** (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. *Dev Biol* 42, 211-221.
**Morin, P. J.** (1999). beta-catenin signaling and cancer. *Bioessays* 21, 1021-1030.
**Muller, H. A., and Wieschaus, E.** (1996). armadillo, bazooka, and stardust are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in Drosophila. *J Cell Biol* 134, 149-163.
**Nagai-Tamai, Y., Mizuno, K., Hirose, T., Suzuki, A., and Ohno, S.** (2002). Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells. *Genes Cells* 7, 1161-1171.
**Nam, S. C., and Choi, K. W.** (2003). Interaction of Par-6 and Crumbs complexes is essential for photoreceptor morphogenesis in Drosophila. *Development* 130, 4363-4372.
**Nam, S. C., and Choi, K. W.** (2006). Domain-specific early and late function of Dpatj in Drosophila photoreceptor cells. *Dev Dyn* 235, 1501-1507.
**Nelson, W. J.** (2003). Adaptation of core mechanisms to generate cell polarity. *Nature* 422, 766-774.

**Newsome, T. P., Asling, B., and Dickson, B. J.** (2000). Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. *Development* 127, 851-860.
**Nusslein-Volhard, C., and Roth, S.** (1989). Axis determination in insect embryos. *Ciba Found Symp* 144, 37-55; discussion 55-64, 92-38.
**Ohno, S.** (2001). Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. *Curr Opin Cell Biol* 13, 641-648.
**Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M., and Hafen, E.** (2002). The Drosophila insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. *Development* 129, 4103-4109.
**Ossipova, O., Bardeesy, N., DePinho, R. A., and Green, J. B.** (2003). LKB1 (XEEK1) regulates Wnt signalling in vertebrate development. *Nat Cell Biol* 5, 889-894.
**Pellikka, M., Tanentzapf, G., Pinto, M., Smith, C., McGlade, C. J., Ready, D. F., and Tepass, U.** (2002). Crumbs, the Drosophila homologue of human CRB1/RP12, is essential for photoreceptor morphogenesis. *Nature* 416, 143-149.
**Pellock, B. J., Buff, E., White, K., and Hariharan, I. K.** (2006). The Drosophila tumor suppressors Expanded and Merlin differentially regulate cell cycle exit, apoptosis, and Wingless signaling. *Dev Biol.*
**Penton, A., Selleck, S. B., and Hoffmann, F. M.** (1997). Regulation of cell cycle synchronization by decapentaplegic during Drosophila eye development. *Science* 275, 203-206.
**Perez-Moreno, M., Jamora, C., and Fuchs, E.** (2003). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* 112, 535-548.
**Petronczki, M., and Knoblich, J. A.** (2001). DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in Drosophila. *Nat Cell Biol* 3, 43-49.
**Pinheiro, E. M., and Montell, D. J.** (2004). Requirement for Par-6 and Bazooka in Drosophila border cell migration. *Development* 131, 5243-5251.
**Polakis, P.** (2001). More than one way to skin a catenin. *Cell* 105, 563-566.
**Povelones, M., Howes, R., Fish, M., and Nusse, R.** (2005). Genetic evidence that Drosophila frizzled controls planar cell polarity and Armadillo signaling by a common mechanism. *Genetics* 171, 1643-1654.
**Qanungo, S., Haldar, S., and Basu, A.** (2003). Restoration of silenced Peutz-Jeghers syndrome gene, LKB1, induces apoptosis in pancreatic carcinoma cells. *Neoplasia* 5, 367-374.
**Quiring, R., Walldorf, U., Kloter, U., and Gehring, W. J.** (1994). Homology of the eyeless gene of Drosophila to the Small eye gene in mice and Aniridia in humans. *Science* 265, 785-789.
**Ready, D. F., Hanson, T. E., and Benzer, S.** (1976). Development of the Drosophila retina, a neurocrystalline lattice. *Dev Biol* 53, 217-240.
**Richard, M., Grawe, F., and Knust, E.** (2006). DPATJ plays a role in retinal morphogenesis and protects against light-dependent degeneration of photoreceptor cells in the Drosophila eye. *Dev Dyn* 235, 895-907.
**Roh, M. H., Fan, S., Liu, C. J., and Margolis, B.** (2003). The Crumbs3-Pals1 complex participates in the establishment of polarity in mammalian epithelial cells. *J Cell Sci* 116, 2895-2906.
**Roh, M. H., Makarova, O., Liu, C. J., Shin, K., Lee, S., Laurinec, S., Goyal, M., Wiggins, R., and Margolis, B.** (2002). The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. *J Cell Biol* 157, 161-172.
**Rolls, M. M., Albertson, R., Shih, H. P., Lee, C. Y., and Doe, C. Q.** (2003). Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. *J Cell Biol* 163, 1089-1098.
**Rossi, D. J., Ylikorkala, A., Korsisaari, N., Salovaara, R., Luukko, K., Launonen, V., Henkemeyer, M., Ristimaki, A., Aaltonen, L. A., and Makela, T. P.** (2002). Induction of cyclooxygenase-2 in a mouse model of Peutz-Jeghers polyposis. *Proc Natl Acad Sci U S A* 99, 12327-12332.
**Sansom, O. J., Reed, K. R., Hayes, A. J., Ireland, H., Brinkmann, H., Newton, I. P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I. S., *et al.*** (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* 18, 1385-1390.
**Sapkota, G. P., Kieloch, A., Lizcano, J. M., Lain, S., Arthur, J. S., Williams, M. R., Morrice, N., Deak, M., and Alessi, D. R.** (2001). Phosphorylation of the protein kinase mutated in Peutz-Jeghers cancer syndrome, LKB1/STK11, at Ser431 by p90(RSK) and cAMP-

dependent protein kinase, but not its farnesylation at Cys(433), is essential for LKB1 to suppress cell vrowth. *J Biol Chem* 276, 19469-19482.
**Sheng, G., Thouvenot, E., Schmucker, D., Wilson, D. S., and Desplan, C.** (1997). Direct regulation of rhodopsin 1 by Pax-6/eyeless in Drosophila: evidence for a conserved function in photoreceptors. *Genes Dev* 11, 1122-1131.
**Shin, K., Straight, S., and Margolis, B.** (2005). PATJ regulates tight junction formation and polarity in mammalian epithelial cells. *J Cell Biol* 168, 705-711.
**Shulman, J. M., Benton, R., and St Johnston, D.** (2000). The Drosophila homolog of C. elegans PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. *Cell* 101, 377-388.
**Small, J. V., and Kaverina, I.** (2003). Microtubules meet substrate adhesions to arrange cell polarity. *Curr Opin Cell Biol* 15, 40-47.
**Smith, D. P., Spicer, J., Smith, A., Swift, S., and Ashworth, A.** (1999). The mouse Peutz-Jeghers syndrome gene Lkb1 encodes a nuclear protein kinase. *Hum Mol Genet* 8, 1479-1485.
**Sotillos, S., Diaz-Meco, M. T., Caminero, E., Moscat, J., and Campuzano, S.** (2004). DaPKC-dependent phosphorylation of Crumbs is required for epithelial cell polarity in Drosophila. *J Cell Biol* 166, 549-557.
**Spicer, J., Rayter, S., Young, N., Elliott, R., Ashworth, A., and Smith, D.** (2003). Regulation of the Wnt signalling component PAR1A by the Peutz-Jeghers syndrome kinase LKB1. *Oncogene* 22, 4752-4756.
**Struhl, G., and Basler, K.** (1993). Organizing activity of wingless protein in Drosophila. *Cell* 72, 527-540.
**Sun, B., Wang, W., and Salvaterra, P. M.** (1998). Functional analysis and tissue-specific expression of Drosophila Na+,K+-ATPase subunits. *J Neurochem* 71, 142-151.
**Sun, T. Q., Lu, B., Feng, J. J., Reinhard, C., Jan, Y. N., Fantl, W. J., and Williams, L. T.** (2001). PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. *Nat Cell Biol* 3, 628-636.
**Suzuki, A., Akimoto, K., and Ohno, S.** (2003). Protein kinase C lambda/iota (PKClambda/iota): a PKC isotype essential for the development of multicellular organisms. *J Biochem (Tokyo)* 133, 9-16.
**Suzuki, A., Hirata, M., Kamimura, K., Maniwa, R., Yamanaka, T., Mizuno, K., Kishikawa, M., Hirose, H., Amano, Y., Izumi, N., *et al.*** (2004). aPKC acts upstream of PAR-1b in both the establishment and maintenance of mammalian epithelial polarity. *Curr Biol* 14, 1425-1435.
**Suzuki, A., Yamanaka, T., Hirose, T., Manabe, N., Mizuno, K., Shimizu, M., Akimoto, K., Izumi, Y., Ohnishi, T., and Ohno, S.** (2001). Atypical protein kinase C is involved in the evolutionarily conserved par protein complex and plays a critical role in establishing epithelia-specific junctional structures. *J Cell Biol* 152, 1183-1196.
**Tabuse, Y., Izumi, Y., Piano, F., Kemphues, K. J., Miwa, J., and Ohno, S.** (1998). Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in Caenorhabditis elegans. *Development* 125, 3607-3614.
**Takeda, H., Miyoshi, H., Kojima, Y., Oshima, M., and Taketo, M. M.** (2006). Accelerated onsets of gastric hamartomas and hepatic adenomas/carcinomas in Lkb1+/-p53-/- compound mutant mice. *Oncogene* 25, 1816-1820.
**Teeter, K., Naeemuddin, M., Gasperini, R., Zimmerman, E., White, K. P., Hoskins, R., and Gibson, G.** (2000). Haplotype dimorphism in a SNP collection from Drosophila melanogaster. *J Exp Zool* 288, 63-75.
**Tepass, U.** (1996). Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of Drosophila. *Dev Biol* 177, 217-225.
**Tepass, U., and Harris, K. P.** (2007). Adherens junctions in Drosophila retinal morphogenesis. *Trends Cell Biol* 17, 26-35.
**Tepass, U., and Knust, E.** (1993). Crumbs and stardust act in a genetic pathway that controls the organization of epithelia in Drosophila melanogaster. *Dev Biol* 159, 311-326.
**Tepass, U., Theres, C., and Knust, E.** (1990). crumbs encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. *Cell* 61, 787-799.
**Tiainen, M., Vaahtomeri, K., Ylikorkala, A., and Makela, T. P.** (2002). Growth arrest by the LKB1 tumor suppressor: induction of p21(WAF1/CIP1). *Hum Mol Genet* 11, 1497-1504.
**Tiainen, M., Ylikorkala, A., and Makela, T. P.** (1999). Growth suppression by Lkb1 is mediated by a G(1) cell cycle arrest. *Proc Natl Acad Sci U S A* 96, 9248-9251.

**Tomlinson, A.** (1985). The cellular dynamics of pattern formation in the eye of Drosophila. *J Embryol Exp Morphol* 89, 313-331.
**Vaccari, T., and Ephrussi, A.** (2002). The fusome and microtubules enrich Par-1 in the oocyte, where it effects polarization in conjunction with Par-3, BicD, Egl, and dynein. *Curr Biol* 12, 1524-1528.
**Vaccari, T., Rabouille, C., and Ephrussi, A.** (2005). The Drosophila PAR-1 spacer domain is required for lateral membrane association and for polarization of follicular epithelial cells. *Curr Biol* 15, 255-261.
**Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E.** (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* 100, 209-219.
**Wang, J. W., Imai, Y., and Lu, B.** (2007a). Activation of PAR-1 kinase and stimulation of tau phosphorylation by diverse signals require the tumor suppressor protein LKB1. *J Neurosci* 27, 574-581.
**Wang, Q., Chen, X. W., and Margolis, B.** (2007b). PALS1 Regulates E-Cadherin Trafficking in Mammalian Epithelial Cells. *Mol Biol Cell* 18, 874-885.
**Wasserscheid, I., Thomas, U., and Knust, E.** (2007). Isoform-specific interaction of Flamingo/Starry Night with excess Bazooka affects planar cell polarity in the Drosophila wing. *Dev Dyn* 236, 1064-1071.
**Watts, J. L., Morton, D. G., Bestman, J., and Kemphues, K. J.** (2000). The C. elegans par-4 gene encodes a putative serine-threonine kinase required for establishing embryonic asymmetry. *Development* 127, 1467-1475.
**Wodarz, A., Grawe, F., and Knust, E.** (1993). CRUMBS is involved in the control of apical protein targeting during Drosophila epithelial development. *Mech Dev* 44, 175-187.
**Wodarz, A., Hinz, U., Engelbert, M., and Knust, E.** (1995). Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. *Cell* 82, 67-76.
**Wodarz, A., Ramrath, A., Grimm, A., and Knust, E.** (2000). Drosophila atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J Cell Biol* 150, 1361-1374.
**Wolff, T., and Ready, D. F.** (1991). The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave. *Development* 113, 841-850.
**Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D.** (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* 13, 2004-2008.
**Xu, D., Wang, Y., Willecke, R., Chen, Z., Ding, T., and Bergmann, A.** (2006). The effector caspases drICE and dcp-1 have partially overlapping functions in the apoptotic pathway in Drosophila. *Cell Death Differ* 13, 1697-1706.
**Xu, T., and Rubin, G. M.** (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* 117, 1223-1237.
**Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S., and Nishida, E.** (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep* 3, 69-75.
**Yamanaka, T., Horikoshi, Y., Sugiyama, Y., Ishiyama, C., Suzuki, A., Hirose, T., Iwamatsu, A., Shinohara, A., and Ohno, S.** (2003). Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. *Curr Biol* 13, 734-743.
**Yamanaka, T., Horikoshi, Y., Suzuki, A., Sugiyama, Y., Kitamura, K., Maniwa, R., Nagai, Y., Yamashita, A., Hirose, T., Ishikawa, H., and Ohno, S.** (2001). PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. *Genes Cells* 6, 721-731.
**Zeng, P. Y., and Berger, S. L.** (2006). LKB1 Is Recruited to the p21/WAF1 Promoter by p53 to Mediate Transcriptional Activation. *Cancer Res* 66, 10701-10708.